Production, Purification and Characterization of Recombinant Viral Proteins

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DOCTOR OF PHILOSOPHY
In
CHEMICAL ENGINEERING

By

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Under the guidance of

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CERTIFICATE

This is to certify that the thesis entitled "**Production, Purification and Characterization of Recombinant Viral Proteins**", being submitted by **Nagesh Kumar Tripathi** for the award of the degree of **Doctor of Philosophy (Chemical Engineering)** is a record of bonafide research carried out by him at the Chemical Engineering Department, National Institute of Technology, Rourkela and Bioprocess Scale up Facility, Defence Research and Development Establishment, Gwalior under our guidance and supervision. The work documented in this thesis has not been submitted to any other University or Institute for the award of any other degree or diploma.

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(Nagesh Kumar Tripathi)

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ABSTRACT

Dengue fever, a mosquito-borne viral disease has become a major worldwide public health problem with a dramatic expansion in recent years. Similarly, Japanese encephalitis (JE) is one of the leading causes of acute encephalopathy affecting children and adolescents in the tropics. There is neither an antiviral therapy nor any effective vaccine available for dengue. Early diagnosis plays a crucial role to forecast an early warning of epidemic and to undertake effective vector control measures for dengue and Japanese encephalitis. Envelope domain III (EDIII) protein is involved in binding to host receptors and it contains specific epitopes that elicit virus neutralizing antibodies.

The objective of the present work is to develop high yield and scalable production process for recombinant dengue and Japanese encephalitis envelope domain III proteins in *Escherichia coli*, purification process to achieve high purity and biologically active protein as well as their characterization for use as diagnostic reagent in enzyme linked immunosorbent assay (ELISA) and possible vaccine candidate molecule. Expression of EDIII proteins of JE and Dengue viruses was carried out in recombinant *Escherichia coli.* Developments of cost effective and simple culture media as well as appropriate culture conditions are generally favorable for large scale production of recombinant proteins. Optimization of culture media was carried out for enhanced production of EDIII protein in *E. coli*. Laboratory scale batch fermentation process in *E. coli* was developed using optimized media and culture conditions. Furthermore, fed-batch fermentation process was also developed in optimized medium. Expression of this protein in *E. coli* was induced with isopropyl β-D-thiogalactoside. The protein was overexpressed in the form of insoluble inclusion bodies (IBs). Cells were disrupted using sonicator or agitator bead mill and IBs were purified. For diagnostic studies, the protein was purified under denaturing conditions using affinity chromatography. The affinity chromatography purified protein was used as an antigen to develop enzyme linked immunosorbent assay (ELISA) to detect antibodies in infected serum and CSF samples.

In order to take this vaccine candidate for further studies, recombinant EDIII protein was produced employing a pilot scale fermentation process. Recombinant EDIII protein expressed as inclusion bodies was solubilized in the presence of urea and renatured by on-column refolding protocol in the presence of glycerol. A three-step purification process comprising of on-column refolding with affinity chromatography, ion-exchange chromatography (IEX) based on salt, and IEX based on pH was developed. The purity of the recombinant EDIII protein was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis, and reactivity of this protein was determined by Western blotting and ELISA. Biological function of the refolded and purified EDIII protein was confirmed by their ability to generate EDIII-specific antibodies in mice that could neutralize the virus. These findings suggest that recombinant EDIII protein is highly immunogenic and elicit high-titer neutralizing antibodies. These results establish the application of these proteins to be used for the diagnosis of JE and Dengue virus infection or for further studies in vaccine development. This process may also be suitable for the high-yield production of other recombinant viral proteins.

Keywords: Dengue. Japanese Encephalitis. Domain III protein. *Escherichia coli.* Media optimization. Bioreactor. Batch fermentation. Fed-batch process. Centrifugation. Cell disruption. Purification. Affinity chromatography. Antibodies. ELISA. Scale up. Pilot scale fermentation. Ultrafiltration. Ion-exchange chromatography. Vaccine.

ABBREVIATIONS

AC	:	Affinity chromatography
APS	:	Ammonium persulfate
BHK-21	:	Baby hamster kidney cells
bp	:	Base pair
BSA	:	Bovine serum albumin
BCA	:	Bicinchoninic acid assay
CIEX	:	Cation ion exchange chromatography
$C_{6/36}$	:	*Aedes albopictus* larvae cell line
DAB	:	Diamino benzidine
DCW	:	Dry cell weight
DF	:	Diafiltration
DIII	:	Domain III
EDIII	:	Envelope domain III
EDTA	:	Ethylenediamine tetracetic acid
ELISA	:	Enzyme linked immunosorbent assay
FPLC	:	Fast Protein Liquid Chromatography
FCA	:	Freund's complete adjuvant
FIA	:	Freund's incomplete adjuvant
FDA	:	Food and drug administration
GFC	:	Gel filtration Chromatography
6x His	:	Hexa histidine tag
HRP	:	Horse radish peroxidase
HBSS	:	Hank's balanced salt solution
HIS	:	Hyper immune sera
IB's	:	Inclusion Bodies
IEX	:	Ion exchange chromatography
IMAC	:	Immobilized metal affinity chromatography
IPTG	:	Isopropyl β-D-thio-galactopyranoside
JEV	:	Japanese encephalitis virus
kDa	:	Kilo dalton
kb	:	Kilo base pairs

LLC-MK2	:	Rhesus monkey kidney cells
LB	:	Luria bertani broth
LPM	:	Liter per minute
mAb	:	Monoclonal antibody
MF	:	Microfiltration
Ni-NTA	:	Nickel-nitrilotriacetic acid
OCR-AC	:	On column refolding with affinity chromatography
OD_{600}	:	Optical Density at 600 nm
OPD	:	o-phenylene diamine dihydrochloride
PAGE	:	Polyacrylamide gel electrophoresis
PBS	:	Phosphate buffered saline
PBS-T	:	Phosphate buffered saline with Tween-20
PCR	:	Polymerase chain reaction
PMSF	:	Phenylmethyl sulfonyl fluoride
PRNT	:	Plaque reduction neutralization test
rDen 1 EDIII	:	Recombinant dengue virus type 1 EDIII
rDen 2 EDIII	:	Recombinant dengue virus type 2 EDIII
rDen 3 EDIII	:	Recombinant dengue virus type 3 EDIII
rDen 4 EDIII	:	Recombinant dengue virus type 4 EDIII
rJEV EDIII	:	Recombinant JE virus EDIII
RT-PCR	:	Reverse transcriptase polymerase chain reaction
SB	:	Super Broth
SDS	:	Sodium dodecyl sulphate
SDS-PAGE	:	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TB	:	Terrific Broth
TEMED	:	N, N, N', N'-Tetramethylethylenediamine
UF	:	Ultrafiltration
Vero	:	African green monkey kidney cell line
WCW	:	Wet cell weight
WHO	:	World Health Organization

LIST OF PUBLICATIONS

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CHAPTER 1

INTRODUCTION

1.1 GENERAL BACKGROUND

Dengue is a mosquito-borne viral disease and has become a major worldwide public health problem. In India, Dengue cases have accelerated enormously with high morbidity and mortality rate in the last two decades (Chaturvedi and Nagar, 2008). As dengue virus has four serotypes, infection with one-serotype results in lifelong immunity to it but there is no cross-protection against the others. Thus, a vaccine must be tetravalent and capable of simultaneously inducing a high level of long-lasting immunity to all four serotypes (Block et al., 2010; Guzman et al., 2010). There is neither a therapeutics nor any effective vaccines available for dengue infection till date. Similarly, Japanese encephalitis (JE) is a major public health problem in Southeast Asia and Western pacific including India. The diagnosis of JE infection is primarily based on symptomatic evaluation of the patients as there is a lack of suitable test for JE infection (Shrivastva et al., 2008; Robinson et al., 2010). The available vaccine for JE is based on inactivated native viral culture which may produce biohazard (Alka et al., 2007; Li et al., 2009). As majority of JE virus infected patients report in rural hospitals with limited facilities, there is a need for a simple and reliable diagnostic test, appropriate for such settings.

Due to unavailability of effective vaccine or therapeutics, early diagnosis plays a crucial role in an early warning of an epidemic, undertake effective vector control measures and patient management also (Abhyankar et al., 2006; Blacksell et al., 2006). Most of the recombinant DNA-based strategies focus on the envelope (E) and nonstructural (NS1) proteins of dengue and JE viruses (Wu et al., 2003; Zhang et al., 2007). The E protein is organized into distinct domains designated as I, II and III. Of these, domain III, stabilized by a single disulfide bond, is particularly important from the viewpoint of diagnostic and vaccine development (Hapugoda et al., 2007; Tan and Ng, 2010). NS1 protein can also be used for early diagnosis of JE and Dengue infections (Lin et al., 2008; Konishi et al., 2009). Recent developments and success in recombinant subunit protein vaccine for several viral diseases opened new opportunities in dengue and JE vaccine research. Keeping in view of the present scenario of severity and spread of dengue and JE, studies on process development and evaluation of new candidate recombinant antigens for possible diagnostic and vaccine development is the need of the hour. Therefore, in the present work we intend to develop a

process for high yield production and to evaluate the diagnostic and vaccine potential of JE and dengue virus envelope domain III proteins.

Recent developments in biochemical engineering and biotechnology involve production of biologicals like vaccines, recombinant proteins, monoclonal antibodies, etc. Demand for these biological products has increased because they are being used in therapeutic, prophylactic and diagnostic purposes (Panda, 2004; Huang et al., 2012). The key component of the commercial success of any biopharmaceutical product is the ability to achieve large-scale production (Buckland, 2005; Huang et al., 2012). The ultimate goal of fermentation research is the cost effective production of desired recombinant protein by maximizing the volumetric productivity (Manderson et al., 2006). *Escherichia coli* is the most commonly used host for heterologous protein production because it is a well-characterized organism in term of genetics, physiology and culture conditions (Shiloach and Fass, 2005). Protein expression level depends on culture conditions, such as medium composition, induction time and inducer concentration, which can be optimized for over-expression of recombinant proteins. Recombinant *E. coli* can be grown to high densities in complex media, semi-defined and defined media (Manderson et al., 2006; Khamduang et al., 2009; Babaeipour et al., 2010). The composition of the growth media is crucial for enhancing product formation (Bhuvanesh et al., 2010). Small scale expression is widely used for optimizing conditions for a large-scale production of recombinant proteins (Mazumdar et al., 2010).

The scale up process of recombinant proteins production may be performed by replacing commonly used shake flasks to lab scale batch or fed-batch fermentations and pilot scale batch fermentations (Bell et al., 2009; Mazumdar et al., 2010). To facilitate the purification of recombinant proteins, the proteins are commonly produced as a fusion proteins that comprise of the protein fused with an affinity tag, such as the hexahistidine tag (Wang et al., 2009; Bhuvanesh et al., 2010). High-level expression of recombinant proteins in *E. coli* often accumulates as insoluble aggregates in the form of inclusion bodies. To recover active protein, inclusion bodies must be solubilized and refolded (Fahnert et al., 2004; Khalilzadeh et al., 2008). Recombinant protein purification using the minimum possible steps is crucial to meet the required level of purity. Immobilized metal ion affinity chromatography (IMAC) has become a well-established and versatile technique for both

analytical and large scale of protein separations. Chemical chaotropes have been traditionally used to solubilize proteins from inclusion bodies (Fahnert et al., 2004). Refolding is usually achieved by removing the chaotrope via buffer exchange after solubilizing the inclusion bodies, using dilution, dialysis or diafiltration. Protein refolding by liquid chromatography is an alternative to the dilution refolding and has been put much highlight in recent years (Wang et al., 2009). IMAC has the potential to perform protein refolding with high recovery of purified recombinant proteins. After the preliminary purification using affinity chromatography, the purity level of these proteins can be further enhanced by ion exchange or size exclusion chromatography. Biologically active and purified protein can be used for evaluation of diagnostic and vaccine potential for dengue and Japanese Encephalitis.

1.2 ORIGIN OF THE PROBLEM

Dengue is an endemic viral disease affecting tropical and subtropical regions around the world. The World Health Organization estimates that there may be 50 million to 100 million cases of dengue virus infections worldwide every year, which result in 250,000 to 500,000 cases of Dengue hemorrhagic fever (DHF) and 24,000 deaths. In India, Dengue hemorrhagic fever and Dengue shock syndrome (DSS) cases have accelerated enormously with high morbidity and mortality rate in the last two decades indicating a serious resurgence of dengue virus infection. There is no suitable treatment for dengue infection and no effective dengue vaccines till date.

Japanese encephalitis (JE) is a major viral encephalitis problem in Southeast Asia with around 50,000 cases and 10, 000 deaths every year affecting mostly children in rural area. The laboratory diagnosis of Japanese encephalitis infection is complicated due to non availability of suitable immunological assays in these areas. Though an inactivated vaccine is available, however its efficacy and coverage is not yet complete.

There is currently a need for developing cost-effective, safe and simple diagnostic test that combines high sensitivity and specificity which may be applicable in both laboratory as well as field conditions. This could as well be used for vaccine studies to control Dengue and JE virus infections. Envelope protein of dengue and JE virus may be very attractive diagnostic and vaccine candidate. The recombinant proteins are to be produced with high yield, by optimization of fermentation conditions. Production of these proteins obviates

expensive and time-consuming virus production in cell culture and the associated biohazard risk. It may be stated that the recombinant protein based kits will be cost effective and surpass other serodiagnostic tests with its applicability in laboratory as well as in field conditions with comparable sensitivity and specificity. Further, large amount of purified protein produced by optimized process may be used in vaccine studies.

1.3 OUTLINE OF THE PRESENT WORK

The present study mainly deals with the production and purification of recombinant proteins of JE and dengue viruses and their characterization for use as a diagnostic reagent to develop detection system for JE and dengue. The outline of this study is:

- Expression of domain III protein of dengue viruses and Japanese Encephalitis (JE) virus domain III and nonstructural 1 (NS1) protein in *E. coli.*
- Optimization of media for production of domain III protein of JE and Dengue virus as well as NS1 protein of JE virus.
- Batch and fed batch fermentation for production of domain III and NS1 proteins in *E. coli.*
- Scale-up of fermentation processes to 100 liters for domain III proteins.
- Development of purification strategy to achieve high purity and biologically active domain III proteins.
- Evaluation of above proteins as a diagnostic intermediate employing ELISA in diagnosis of Dengue and JE infections.
- Determination of biological activity of domain III protein of JE virus for their possible vaccine potential.

1.4 ORGANIZATION OF THE THESIS

This thesis contains ten chapters. The present chapter, chapter 1 is an introductory chapter. In chapter 2, a detailed review of the literature pertinent to the previous works done in this field has been listed. Chapter 3 presents the materials selection for the experiments, methods for the production, purification and characterization of recombinant proteins. In chapter 4, production of recombinant JE virus envelope domain III protein by batch and fed-batch fermentation as well as its diagnostic potential is summarized. Chapter 5 presents the

result of pilot scale production, refolding with purification and vaccine potential of recombinant JE virus envelope domain III protein. Chapter 6 presents production, purification and evaluation of diagnostic potential of recombinant JE virus nonstructural 1 protein. In chapter 7, production of recombinant dengue virus type 3 envelope domain III protein by batch and fed-batch fermentation as well as its diagnostic potential is summarized. Chapter 8 presents the result of pilot scale production, refolding with purification and vaccine potential of recombinant dengue virus type 3 envelope domain III protein. In chapter 9, process development for production of recombinant dengue virus serotype 1, 2 and 4 envelope domain III protein is described. The use of tetravalent dengue virus envelope domain III protein as a diagnostic reagent in ELISA is also presented in chapter 9. Finally, in chapter 10, the conclusions drawn from the above studies are described. There is also a brief note on the scope for further study in this field.

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CHAPTER 2

LITERATURE REVIEW

2.1 RECOMBINANT PROTEIN PRODUCTION

Biotechnology is defined by the United Nations as "any technological application that uses biological systems, living organisms or derivatives thereof, to make or modify products or processes for specific use". The demand for therapeutic recombinant proteins is set to see a significant increase over the next few years. As a consequence, the processes used to produce these proteins must be able to meet market requirements (Huang et al., 2010). Recombinant proteins have gained enormous importance for clinical applications. Nearly 30% of currently approved recombinant therapeutic proteins are produced in *Escherichia coli* (Huang et al., 2012). The production of recombinant proteins has become a huge global industry with an annual market volume exceeding $50 billion (Schmidt, 2004). The global biologics market valued at an estimated $149 billion in 2010 and is expected to reach $239 billion by 2015 (http://www.scribd.com/doc/90133606/Biologic-Therapeutic-Drugs-Technologies-and-Global-Markets).

Molecular biology offers technologies whereby proteins can be produced and purified easily and more efficiently than ever before. Using recombinant DNA techniques such as gene fusion it is possible to generate chimeric proteins, which are novel in structure and function. The protein engineering has become a powerful tool in molecular biology to investigate protein function, in addition to production and purification of useful proteins (Sassenfeld, 1990; Sahdev et al., 2008). The main applications of recombinant proteins obtained by genetic engineering are in the medical therapeutic fields (e.g., production of recombinant vaccines, and therapeutic proteins for human diseases), and medical diagnosis (e.g., antigen engineering for poly and monoclonal antibody production used in disease testing). Other areas where recombinant proteins are commonly utilized include enzymes for food and fiber production, testing food for microbial contamination and veterinary medicine (Nilsson et al., 1992; Huang et al., 2012). Most proteins are expressed in infinitesimal amounts in their native cells and tissues, and it is only by recombinant techniques that it is possible to produce amounts great enough for basic research or for practical uses. Therefore, the expression of engineered proteins in efficient heterologous protein expression system is integral to the production, and purification of many proteins of interest.

2.2 RECOMBINANT PROTEIN PRODUCTION IN *E. COLI*

Demands of the expanding biotechnology industry have driven to different improvements in protein expression technology, which have been translated into the production of a spectrum of recombinant proteins in different systems for a wide variety of purposes. Most of the recombinant proteins are now-a-days produced either in bacteria, yeasts, engineered animal cell lines, hybridoma cells or even human cells. However, research continues on the development of alternative production systems, particularly in the use of transgenic animals or plants (Walsh, 2005; Desai et al., 2010). In the recent years, baculovirus and mammalian cell cultures have gained importance for the production of biopharmaceuticals due to the increasing needs of complex proteins and antibodies. An alternative to baculovirus or mammalian expression systems are yeasts, especially when large amounts of secreted protein are required (Porro et al., 2011). If the product contains post-translational modifications, *Saccharomyces cerevisiae* or *Pichia pastoris* may offer an economic alternative because they can grow to high cell densities using minimal media (Mattanovich et al., 2012), but the use of yeasts is limited due to their inability to modify proteins with human glycosylation structures for the production of therapeutic glycoproteins (Porro et al., 2011). The use of transgenic plants and animals as production vehicles may also play a role in applications requiring exceptional product volumes, but regulatory issues still remain to be addressed.

Even though the choice of expression system is progressively widening, *E. coli* is still the dominant host for recombinant protein production. It is used in many industry fields to produce high value intermediates, detergents, nutraceuticals and pharmaceuticals, amongst others. It is the most popular choice when simple proteins are required, and significant advances have been also made to overexpress complex proteins, hormones, interferons and interleukins in it (Walsh, 2005; Tripathi et al., 2009). The production of heterologous proteins or parts thereof in cytoplasmic compartments of *Escherichia coli* offers multiple applications, for example, in diagnostics and vaccine development (Panda, 2004; Huang et al., 2012). Thus bacterial expression systems are the preferred choice for production of many recombinant proteins. The reasons for this lie in the cost-effectiveness of bacteria, their well-characterized genetics, and the availability of many different bacterial expression systems. As a host for recombinant expression, *E. coli* is especially valued

because of its rapid growth rate, capacity for continuous fermentation, low media costs and achievable high expression levels (Yin et al., 2007; Kamionka, 2011). Foreign proteins can be produced in *E. coli* in large amounts (5-50% of total protein). The major drawbacks of using *E. coli* for recombinant protein production are its lack of secretion systems for efficient release of proteins to the growth medium, limited ability to facilitate extensive disulfide-bond formation and othe posttranslational modifications, inefficient cleavage of the amino terminal methionine which can result in lowered protein stability and increased immunogenicity, and occasional poor folding due to lack of specific molecular chaperones (Yin et al. 2007; Berkmen, 2012). Even though *E. coli* may not be useful for all foreign protein production, it has been successfully utilized to produce many functional human proteins such as human growth hormone, proinsulin, interferon-gamma and antibody fragments (Schmidt, 2004; Tripathi et al., 2009). Important bioproducts produced in *E. coli* are listed in table 2.1.

Table 2.1. List of bioproducts in *E. coli* with their manufactures.

Product	**Host**	**Company**
Asparaginase	*E. coli*	Merck
r Cholera toxin B subunit	*E. coli*	SBL vaccine
rh B-type natriuretic peptide	*E. coli*	Scios/Johnson & Johnson
Tissue plasminogen acticator	*E. coli*	Roche
rh Insulin	*E. coli*	Eli Lilly, Aventis
rh Growth hormone	*E. coli*	Genentech, Eli Lilly
h Parathyroid hormone	*E. coli*	Eli Lilly
rh Interferon α-2a and 2b	*E. coli*	Hoffmann-LaRoche, Schering
Interferon alfacon-1	*E. coli*	Valent
r IL-2 diptheria toxin fusion	*E. coli*	Seragen/Ligand
rh IL-1 receptor antagonist	*E. coli*	Amgen
r IL-2	*E. coli*	Chiron
r Interferon β-1b	*E. coli*	Schering AG, Chiron
r Interferon γ-1b	*E. coli*	Genentec

2.2.1 *Escherichia coli* as a Host

Escherichia coli (Figure 2.1) is a Gram-negative, facultative anaerobe and non-sporulating bacterium that is commonly found in the lower intestine of warm-blooded animals. The morphology of cells is rod-shaped, about 2 micrometers long and 0.5 micrometers in diameter. Most of the *E. coli* strains are harmless, but others can cause serious damage to humans. The harmless strains are part of the normal flora of the gastrointestinal tract, and can benefit their hosts by producing vitamin K2 and by preventing the establishment of pathogenic bacteria within the intestine (Bentley and Meganathan, 1982). However, *E. coli* can be easily grown outside the intestine as well, and it has become a model organism in biotechnology because of the simplicity of its genetics and manipulation.

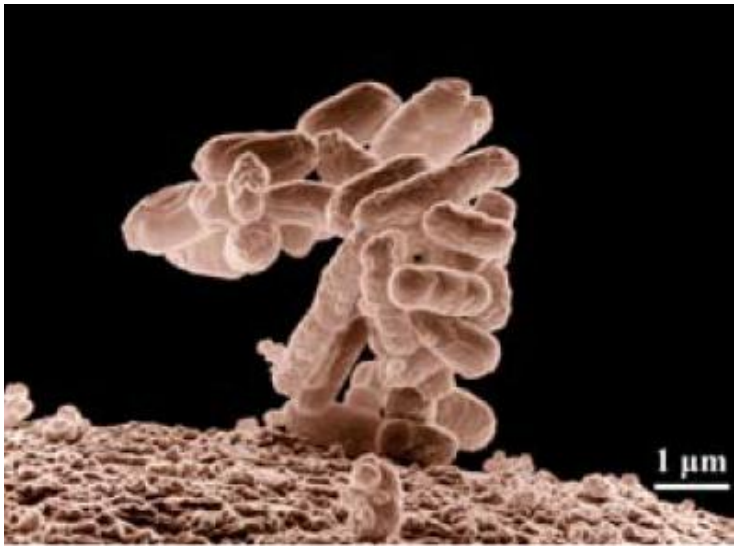

Figure 2.1. Escherichia coli cells electron micrograph (magnified 10.000 times).

Unlike eukaryotes, the bacterial chromosome is not enclosed inside of a membrane-bound nucleus but instead resides inside the bacterial cytoplasm. In there, the chromosome exists as a compact (usually circular) supercoiled structure which can be easily modified. To date, the most popular target for genetic manipulation in *E. coli* has been the modification of host cell metabolism to reduce acetate formation (Eiteman and Altman, 2005). Other genetic manipulations have been focused on improved protein folding (which may be achieved by overexpression of intracellular chaperones), or efficient disulfide bond formation (Berkmen, 2012). All these modifications have driven to the

existence of various strains of *Escherichia coli* with different genotypes (genetic constitution) which are used as expression systems. In particular, *E. coli* K12 and *E. coli* B (e.g. BL21 or BLR) and their many derivatives are the most commonly used hosts (Sahdev et al., 2008; Tegel et al., 2010).

All these features allow *E. coli* to be one of the most competitive hosts for rapid and economical production of simple recombinant proteins, amino acid and metabolite production when compared to other hosts. Commercial products mainly include recombinant proteins which are considered low-volume high-value products (Riesenberg et al., 1990). However, recent advancements in metabolic engineering made it possible to use *E. coli* as a platform to produce high-volume low-value products. In all these processes, high cell density and high volumetric yields are essential for economical feasibility. Capital investment and operation costs are reduced due to size reduction of fermentation equipment, upstream utilities, downstream units, etc. In this sense, process development plays an important role to maximise target product yields and to minimise production costs.

2.2.2 Expression Systems for *Escherichia coli*

Various *E. coli* expression systems are available commercially and in the public domain, which is shared within the scientific community. Each system offers different benefits for protein expression, detection, and purification, and should be considered according to the specific criteria and requirements each protein poses. In the industry, the selection of the host cell will be strongly influenced by the type and use of the product, as well as economic or intellectual property issues. The most popular commercial expression system for *E. coli* is the Novagen pET expression system based on the T7 promoter (Studier et al., 1990; Li et al., 2011). Systems using the λpL promoter/cI repressor (e.g., Invitrogen pLEX), Trc, Tac promoter (e.g., GE pTrc, pGEX), ara*BAD* promoter (e.g., Invitrogen pBAD) and hybrid lac/T5 (e.g., Qiagen pQE) promoters are common (Graumann and Premstaller, 2006). The selection and design of the expression plasmids influence synthesis rates, plasmid copy number, the segregational plasmid stability and therefore productivity and regulatory issues. For industrial applications, selective pressure by antibiotics is mainly maintained in pre-cultures, main cultures are usually grown without selective pressure. The ideal expression vector combines medium to high copy numbers with tight regulation of gene

expression to achieve rapid cell growth to high densities before the induction phase (Jana and Deb, 2005; Sahdev et al., 2008).

2.3 CELL GROWTH AND FERMENTATION

When using *E. coli* as expression system, high cell density cultures are usually carried out to increase target protein yields. Main factors which affect high density growth of *E. coli* are solubility of solid and gaseous substrates in water (limitation/inhibition of substrates with respect to growth may occur), accumulation of products or metabolic by-products to inhibitory levels, high evolution rates of CO_2 and heat as well as increasing viscosity of the medium (Riesenberg, 1991; Shiloach and Fass, 2005). Since this work was developed at bench and pilot scale, neither heat transfer or high power consumption due to high viscosity of the medium nor the utilisation of pure oxygen were problems, but *E. coli* growth can be also limited/inhibited by nutritional requirements including carbon, nitrogen, phosphorus, sulphur, magnesium, potassium, iron, manganese, zinc, copper and other growth factors (Tripathi et al., 2009). For high cell density cultures, the nutritional requirements can not be added initially to the basal media, since most media ingredients become inhibitory to *E. coli* when added at high concentrations. Another problem is the precipitation of media components that can hamper adequate supply, interfere with the fermentation process and monitoring devices and affect downstream recovery and purification processes. Therefore, a well designed media and feeding strategy are essential to achieve high cell density cultures. Another motivation to develop different feeding strategies was to decrease acetate accumulation. Acetate accumulation can be avoided in stirred tank reactors by means of culture medium design and methods for carbon source feeding (Eiteman and Altman, 2005).

To produce recombinant proteins in large quantities, fermentation technology is generally applied to increase cell density and protein productivity. Fermentation provides control over key chemical, physical and biological parameters that affect cell growth, as well as recombinant protein production. These include, but are not limited to, temperature, dissolved oxygen (DO) level, pH and nutrient supply. Fermentation can be classified into three groups: batch, fed batch, and continuous (Yee and Blanch, 1992; Stanbury et al., 1999). These methods can achieve cell concentrations about 100 g/l of dry cell weight and can provide cost-effective production of recombinant proteins. The development and design of

fermentation process itself play a key role for achieving productivity and robustness at scale (Schmidt, 2005). A robust large scale fermentation process would also need to consider the composition and cost of the media, feeding strategies and scale-up process.

2.3.1 Batch Fermentation

Batch fermentation is an easy way to culture cells to reach high cell density in a very short time (Bhuvanesh et al., 2010). However, due to its low productivity compared to fed-batch culture, it is usually used to produce protein for the purposes of protein characterization or toxicology study. If a therapeutic protein is to be used in a low-dose therapy or is an orphan drug, a batch process is a good choice to ensure efficient and reliable production (Huang et al., 2012).

2.3.2 High Cell Density Fed-batch Fermentation

High cell density culture (HCDC) techniques for culturing *E. coil* have been developed to improve productivity, and also to provide advantages such as reduced culture volume, enhanced downstream processing, reduced waste water, lower production costs and reduced investment in equipment. High cell-density culture systems also suffer from several drawbacks, including limited availability of dissolved oxygen, carbon dioxide levels which can decrease growth rates and stimulate acetate formation, reduction in the mixing efficiency of the fermentor, and heat generation (Lee, 1996; Panda, 2003). The reduction in acetate accumulation caused a significant improvement in the production of recombinant protein. Mutant strains of *E. coli* that are deficient in other enzymes have also been developed and shown to produce less acetate and higher levels of recombinant proteins. In high cell density fermentation, maximizing cell concentration helps in increasing the volumetric productivity of recombinant proteins (Fong and Wood, 2010). It is also essential that cell growth be achieved in optimal time period to improve the overall production of the recombinant protein. Toxicity of acetate, slow growth rate, instability of plasmid, depletion of amino acid pools to sustain high rate of protein synthesis affect the specific cellular yield of recombinant protein at high cell concentration (Babu et al., 2000; Manderson et al., 2006; Tripathi et al., 2009). It is expected that by analyzing all these parameters during high cell density fed- batch growth of *E. coli* will lead to high volumetric production of the desired protein.

2.4 IMPORTANT PARAMETERS AFFECTING FERMENTATION PROCESS

The operating condition such as pH, temperature and more importantly O_2 supply is very very essential for supporting high cell growth. Solubility of oxygen in medium is very low and with increase in cell concentration during fed-batch growth, the solubility is reduced. At very high cell concentration use of air does not suffice the respiratory demand of the rapidly growing *E. coli* cells (De Leon et al., 2003). Increasing aeration rate, feeding O_2 rich air, decreasing temperature, increasing partial pressure of the culture vessel are some of the method employed to maintain aerobic condition during cell growth. It has been widely documented that oxygen not only influences the cell growth but also has effect on gene expression by influencing the oxidative status of many enzymes (Castan and Enfors, 2000). Hence it's essential that along with proper feeding of nutrients, supply of oxygen should be at optimal level to support good growth and provide oxidizing environment for quality protein synthesis (Chen et al., 1995; De Mare et al., 2005). Composition of medium, physical parameters during growth and operating conditions are the most important factor that influences the cell growth. Limitation and or inhibition of substrates, limited capacity of oxygen supply, formation of metabolic byproducts and instability of plasmid during long hours of cultivation are most of the problems encountered during high cell density growth of *E. coli* (Schmidt, 2005; Tripathi et al., 2009). These, most of the times depends on host strain, vector and strength of promoter. Dense culture requires large amounts of O_2 to support good growth and thus necessitates unconventional aeration strategy to maintain dissolved oxygen concentration at a suitable level throught out the growth period. In most of the cases of *E. coli* being used as a host for recombinant protein, the production phases start after induction with suitable inducer (Panda, 2003; Fong and Wood, 2010). Thus in principle, growth phase and production phase can be delineated in the same vessel for the high volumetric yield of the recombinant protein. However, many times operation of reactor during cell growth influence the specific yield of the recombinant proteins. Thus while developing fed-batch operation to increase unit cell growth in the reactor it is equally essential to take care of the factors which affect the specific yield of the recombinant protein.

2.4.1 Development of Growth Media

The composition of the cell growth medium must be carefully formulated and monitored, because it may have significant metabolic effects on both the cells and protein production. For example, the translation of different mRNAs is differentially affected by temperature as well as changes in the culture medium. Among the three types of media defined, complex and semi-defined; defined media are generally used to obtain high cell-density and can be controlled during culture (Berger et al., 2011). However, semi-defined or complex media are sometimes necessary to boost product formation. Chemically defined media are generally known to produce slower growth and low protein titres than complex media (Zanette et al., 1998). Nonetheless, use of complex media in producing recombinant proteins is a common practice because these media attain more consistent titres, allow easier process control and monitoring, and simplify downstream recovery of the target protein (Manderson et al., 2006; Bhuvanesh et al., 2010). Some nutrients, including carbon and nitrogen sources, can inhibit cell growth when they are present above a certain concentration. This explains why just increasing the concentrations of nutrients in batch culture media does not yield high cell-density.

Optimization of medium components for enhanced production of recombinant protein is also a common practice in industry. One simple way to accomplish this goal is to modify the published media recipes, when high cell growth and protein expressions have been demonstrated (Huang et al., 2012). To grow cells in a high density, it is necessary to design a balanced nutrient medium that contains all the necessary components in supporting cell growth avoiding inhibition. It is desirable to make the feed-solution as simple as possible by including sufficient non-carbon and non-nitrogen nutrients in the starting medium (Volonte et al., 2010). One of the essential requirements during fed-batch operation is to supply nutrients to promote cell growth (Khalilzadeh et al., 2004). To limit their toxicity to the growing cells nutrients such as glucose, glycerol, ammonia, salt are fed approximating their requirement. Accumulation of nutrients at high concentration inhibits growth and recombinant protein expression. High glucose causes Crabtree effect and leads to accumulation of acetate which is inhibitory to cell growth (Panda, 2003). In general most of the medium used for high cell growth of *E. coli* have carbon source mostly glucose or glycerol, major salts like phosphate, sodium, potassium, magnesium, ammonia and sulphates,

iron, minor trace elements and complex nitrogenous. High-density growth in general is initiated with low concentration of most required substrate and the nutrients are added later in the growth period (Fahnert et al., 2004; Babaeipour et al., 2008). Ideally components should be added to the fermentor at the same rate at which they are consumed so that it prevents the nutrient accumulation in toxic level while promoting good growth. Another factor which needs attention during medium formulation is the solubility of many components particularly while making concentrated solution for fed-batch addition. High concentration of glycerol, glucose, yeast extract and trace elements needs careful composition to avoid precipitation.

It has been observed that addition of yeast extract with glycerol not only help in reducing secretion of acetic acid during growth of *E. coli* but also help in utilization of acetic acid during carbon limitation. Apart from this, organic nitrogen source like yeast extract and soybean hydrolysate have been reported to enhance the specific cellular yield of the expressed protein particularly during high cell density fermentation where the demand of nitrogenous source become very high following induction (Panda, 2003; Mazumdar et al., 2010). Presence of yeast extract in the medium also help in lowering the inhibitory effect of acetic acid and also work as a better physiological buffer in comparison to the minimal medium (Manderson et al., 2006). Therefore, its use in the feeding medium along with glycerol or glucose helped in avoiding the need of complex genetic manipulation to lower the acetic acid secretion. This indicated that with the use of yeast extract in the feeding medium not only the specific yields of protein can be maintained in high cell density fermentation but also the duration of the process can be reduced resulting in high volumetric productivity of the expressed protein (Zhang et al., 2010).

2.4.2 Feeding Strategy during Fermentation Process

Feeding of nutrient is critical to the success of fed-batch process, as it not only affects the maximum attainable cell concentration, but also cell productivity. The use of fed-batch cultures has been shown to significantly increase the cell-density and specific protein production by overcoming inhibitory substrate concentrations encountered in batch culture (Chen et al., 1995). Various strategies exist for controlled feeding of fed-batch cultures. From a process engineering point of view, there are two principal strategies for the control of nutrient feed: the open-loop and the closed-loop (feedback) control. The choice of the nutrient feeding strategy has been done depending on the expression system, since no any

strategy is suitable in this case. Process optimization performed in present work required the development of different nutrient feeding strategies depending on both the expression system and target protein which were considered.

A pH-stat feeding system monitors the pH of the fermentation for indications of acetic acid production. The feed rate is increased until the maximum growth rate is reached as indicated by a metabolic overflow causing production of acid and a consequent decrease in pH. The DO-stat operation relies on the fact that specific oxygen uptake reaches a maximum at the maximum growth rate. Changes in oxygen uptake rate following a pulse of feed are used to determine whether the microorganism is at its maximum growth rate. Exponential feeding makes use of an empirical model of growth, to regulate the feed rate (Lee, 1996; Manderson et al., 2006; Mazumdar et al., 2010). One of the essential requirements to obtain good cell growth during cultivation is to supply nutrients in a manner that is desirable. Ideally, by providing proper nutrient and operating conditions, exponential growth of *E. coli* can be maintained so that high cell concentration is achieved in less time. Oxygen supply, saturation of the oxidative capacity of cells at high glucose concentration, build-up of acetate to toxic level, plasmid instability and low productivity associated with cell growth at high specific growth rate has led to the development of different feeding strategy to achieve high cell growth at reasonable time (Babaepour et al., 2008). Direct feedback control is also possible by measuring on-line concentration of the growth-limiting substrate in the culture broth and adjusting the concentration to the preset value by automatic feeding. The feeding solution is added either simply in a constant way or by an exponential feeding programme. Two points are important to guarantee a good yield: (a) the flow of the feed solution must be low enough to allow carbon source limitation in the fermenter; (b) the flow of the feed solution must be regulated in a way that the specific growth rate does not decrease below 0.1 h^{-1} until the point of induction.

2.4.3 Induction Strategy and Effect of Oxygen during Fermentation Process

Along with chromosomal DNA, most bacteria also contain small independent pieces of DNA called plasmids which are capable of replicating independently of the chromosome (Figure 2.2). Plasmids (or expression vectors) often encode for genes that are advantageous but not essential to their bacterial host, and can be easily gained, lost or transferred by cells. These properties make them basic tools in biotechnology: they are used

to multiply particular genes or to make large amounts of proteins. In the latter case, bacteria containing a plasmid harbouring the gene of interest are grown and, then, transcription of the inserted gene can be induced to produce the target protein.

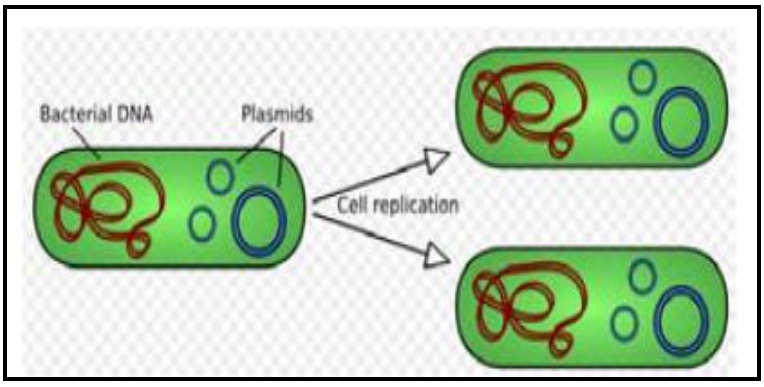

Figure 2.2. Plasmid replication scheme.

In order to control recombinant protein expression in *E. coli*, several inducible promoters have been developed. When using them, induction of the target gene transcription is controlled by a signal which will depend on the type of promoter. Idealistically, the promoter should not allow expression before induction, and should allow adjustable induction of protein expression. The most commonly used inducer for T7 promoter systems is isopropyl-β-D-thiogalactopyranoside (IPTG) (Figure 2.3). This synthetic inducer is expensive and may interfere with cell growth at high concentration. Owing the cost of IPTG, it would be advantageous to use less inducer to obtain the same level of transcription (Hansen et al., 1998).

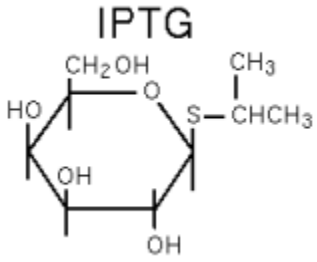

Figure 2.3. Isopropyl-β-D-thiogalactopyranoside molecule.

Expression systems used in this work contained vectors with T7 and T5 promoters. Hence, IPTG was used as inducer. Cellular responses to induction depend on a number of

interacting factors including the host/vector system and properties of the expressed protein. Therefore, the timing of induction of new recombinants needs to be empirically determined for each new clone (Manderson, 2006; Tripathi et al., 2009). Inducer concentration trials required since IPTG inducer is expensive. The concentration of IPTG required for complete induction is known to vary widely along with various clones expressing different proteins. The induction strategy needs optimization to maintain specific cellular protein yield during high cell density fed-batch fermentation. In fed-batch culture separation of the two phases can be achieved by delaying induction of the culture until the culture has completed its growth to the required high densities. Considering these aspects, it is essential to decide the induction time so that both cell growth and protein yield are maximized.

In recent times the secondary role of oxygen on maintenance of cell physiology and quality of the recombinant protein has been a major concern for the high volumetric yield of recombinant protein from *E. coli*. Fluctuations in oxygen contents during high productive fermentation process can cause oxidative stress within the cells leading to limitation in amino acid production, plasmid instability and more importantly oxidation of proteins (Lee, 1996; Panda, 2003). These effects altogether may affect the quality of the final product. Oxygen often becomes limiting in HCDCs owing to its low solubility. The saturated dissolved oxygen (DO) concentration in water at 25°C is ~7 mg/l but oxygen supply can be increased by increasing the aeration rate or agitation speed (Lee, 1996). Oxygen-enriched air or pure oxygen has also been used to prevent oxygen limitation as for example in high yield production of recombinant malaria antigen and human interferon (Yazdani et al., 2004; Babaeipour et al., 2008). However, pure oxygen is expensive and increases production costs when used in large quantities. As oxygen consumption increases with growth rate, the oxygen demand of cells can be reduced by lowering the growth rate.

2.4.4 Scale up of Fermentation Process

For commercial production of recombinant proteins, the fermentation usually starts in a laboratory scale bioreactor (e.g., 5–30 l) to identify suitable growth and protein expression conditions. The process then transfers to pilot level (e.g., 40–600 l) to establish optimal operating parameters and finally to manufacturing scale (e.g., over 2,000 l) to reach high productivity. The scale-up process for any recombinant protein should aim for high productivity with consistency in the protein quality and specific yield (Ravi et al., 2008; Bell

et al., 2009). However, as the scale increases important biological, chemical and physical parameters affecting cell growth, as well as protein expression, will also change. This makes the scale-up process a challenging task. The common problems associated with scale-up originate from poor mixing, which increases circulation time and creates stagnant regions. This leads to imbalanced and zonal distribution of oxygen, nutrients, pH, heat and metabolites inside the bioreactor (Schmidt, 2005; Lee, 2009). Therefore, several strategies have been used as principles to scale up *E. coli* fermentation to minimize the differences between scales by keeping one or more parameters constant from laboratory to pilot and plant scale bioreactors. These parameters include power input per liquid volume (P/V), oxygen transfer rate (OTR), oxygen mass transfer coeffcient (k_{La}), impeller tip speed, mixing time and impeller Reynold's number (N_{Re}) (Junker, 2004; Garcia-Ochoa and Gomez, 2009). Traditionally, the constant P/V has been shown to be a successful scale-up criterion for large scale fungal and mammalian cell fermentation, but may be limited to recombinant *E. coli* culture because of its high energy requirement (Huang et al., 2012).

Development of sensors for DO measurement attributed that scaling up based on constant DO concentration is the most appropriate approach for microorganisms, such as *E. coli* growing under aerobic conditions, and other studies have shown that it is indeed the most applied scale-up parameter (Junker, 2004; Garcia-Ochoa and Gomez, 2009). The k_{La} is directly related to a bioreactor's configuration and can be modulated by manipulating the bioreactor's agitation speed, impeller design and air/O_2 flow rate. In a large-scale and high-performance *E. coli* fermentation where power input and mixing are not an issue, a similar OTR and heat transfer rate will normally be used to ensure high productivity (Schmidt, 2005). Scale-up based on a constant mixing time or tip speed usually has a higher success rate when the scale-up factor is small (4–40 l, scale-up factor 10) (Wu et al., 2009; Huang et al., 2012). In practice, however, the important scale-up parameters for each product may be different because of process differences and the operation limitations imposed by manufacturing facilities (Tripathi et al., 2009). Therefore, a detailed and comprehensive process characterization must be carried out in advance to identify critical parameters influencing protein yield and quality. Those parameters then should be kept constant during the scale-up processes. The knowledge gained is then used to optimize the fermentation processes to improve the robustness of the process in a scale-up practice.

2.5 PURIFICATION STRATEGIES FOR RECOMBINANT PROTEINS

Refolding and purification of bioactive protein is the major bio-processing parameter for efficient production of prophylactic, diagnostic or therapeutic proteins from *E. coli*. As level of expression for soluble proteins are in general low in *E. coli*, rarely such process is employed for large-scale production of therapeutic protein. High level expressions of protein in the form of inclusion body are generally used for large-scale production of therapeutic protein (Fahnert, 2004). The formation of inclusion bodies in *E. coli* facilitates the isolation of the protein of interest from the cytoplasm at the cost of its native structure. In general inclusion bodies are solubilized by the use of high concentration of denaturants such as urea or guanidine hydrochloride, along with a reducing agent such as DTT or β-mercaptoethanol (Wang et al., 2007). The solubilized proteins are then refolded by slow removal of the denaturant in the presence of oxidizing agent. In most of the therapeutic recombinant protein cases, the yield of bioactive protein from the inclusion bodies is around 15-25 % of the total expressed protein. The overall process yield and economic viability of the recombinant *E. coil* fermentation process mostly depend on the efficient recovery of bioactive protein from the inclusion bodies (Esfandiar et al., 2010). It is thus necessary to have information about the solubilization and refolding of inclusion body protein from *E.coli.*

2.5.1 Recombinant Protein as Inclusion Bodies

The formation of inclusion bodies is mainly attributed to the overexpression of recombinant proteins in the cell lacking required accessories for its folding to native form. There is no direct correlation between the propensity of the inclusion body formation of a certain protein and its intrinsic properties, such as molecular weight, hydrophobicity, folding pathways and so on (Panda, 2003; Garcia, 2010). In case of proteins having disulfide bonds, formation of protein aggregation as inclusion bodies is anticipated since the reducing environment of bacterial cytosol inhibits the formation of disulfide bonds (Berkmen, 2012). The consequences result in improper folding of the protein in aggregation in to inclusion bodies. Although protein expression in the form of inclusion body is often considered undesirable, their formation can be advantageous, as their isolation from cell homogenate is a convenient and effective way of purifying the protein of interest (Babu et al., 2000). Converting this inactive misfolded inclusion body protein in to soluble active form can result in high recovery of recombinant protein.

2.5.2 Inclusion Body Formation, Isolation and Solubilization

Inclusion bodies are dense particles of aggregated protein found in both cytoplasmic and periplasmic space of *E. coli* during high level expression of foreign protein (Jurgen et al., 2010). The exact reason for protein aggregation in to inclusion body formation is not clear yet. There could be several possible reasons for the intracellular aggregation of the recombinant protein and the predominant ones could be due to (1) high local concentration of protein synthesis in cytoplasm (2) lack of cellular compartmentation and oxidizing environment the *E. coli* cell thus preventing S-S bond necessary for proper folding (3) lack of mammalian post translational modifying enzymes and foldases (PDI, PPI and chaperone system) during high level expression of protein (4) aggregation behavior of the intermediates of the protein folding path ways (Singh and Panda, 2005). The propensity to form insoluble aggregates does not correlate with other factor such as size of the expressed protein and use of fusion construct. Recombinant protein deposition in inclusion body is commonly observed with hydrophobic proteins as hydrophobic interaction among the partially folded protein molecules have been found to be responsible for aggregation in to inclusion bodies (Jurgen et al., 2010). Significant features of protein aggregates in inclusion bodies are the existence of native-like secondary structure of the expressed protein and their resistance to proteolytic degradation. Analysis of the secondary structure of β-lactamase inclusion bodies from *E. coli* by Raman spectroscopy indicated the presence of amide bond, thus indicating the existence of native like protein structure in the inclusion body protein (Panda, 2003; Fahnert, 2004).

The formation of inclusion bodies thus facilitates the easy isolation and recovery of the expressed proteins in denatured form (Rodriguez et al., 2010; Peternel and Komal, 2010). As the inclusion bodies have high density (~ 1.3 mg ml $^{-1}$), these are easily separated by high-speed centrifugation after cell disruption. The most common process for cell lysis is sonication, bead milling and high-pressure disruption following a lysozyme treatment. Further purification of inclusion bodies can be achieved by washing with detergents, low concentration of salt and or urea (Dasari et al., 2008). With proper isolation and washing process, more than 80% pure inclusion body can be prepared from *E. coli*. Recently ultrafiltration using membrane of different pore size has been used for isolation of inclusion bodies from *E. coli* cells (Ledung et al., 2009) however centrifugal isolation have been found to be the best method for isolating the inclusion bodies from that of the membrane or cellular components.

In general, proteins expressed as inclusion bodies are solubilized by the use of high concentration (6-8 M) of chaotropic solvents. Chaotropic agents such as urea, guanidine hydrochloride, and detergents such as SDS along with reducing agents like β-mercaptoethanol, dithiothreitol or cysteine have been extensively used for solubilizing the inclusion body proteins (Wang et al., 2006). In many cases of inclusion body solubilization, use of reducing agent like DTT, 2-mercaptoethanol improves the solubilization yield in the presence of chaotropic agents. This helps in maintaining cysteine residue in reduced state and thus prevent non-native intra or inter disulfide bond formation in highly concentrated protein solution at alkaline pH. Chelating agents like EDTA are frequently used in the solubilization buffer to prevent metal catalyzed air oxidation of cysteines (Dasari et al., 2008). Use of extreme pH with combination of low concentration of denaturing agent or temperature has also been used for solubilization of inclusion body protein (Singh and Panda, 2005; Li et al., 2012). The choice of the solubilizing agent greatly affects the refolding yield and cost of the overall process.

2.5.3 Refolding of Solubilized Recombinant Protein

The soluble proteins in general are refolded in to their native state after removing the chaotropic agents or other salts by dialyzing the proteins in buffers containing reducing and oxidizing agents (Wang et al., 2006; Tan et al., 2010). Purification of the recombinant protein either can be carried out before renaturation in denaturing conditions or after refolding of the solubilized protein. Refolding followed by purification is generally preferable as some of the high molecular aggregates along with the contaminants can be co-purified in single step. Dilution of the solubilized protein directly in to the renaturation buffer is the most commonly used method for small scale refolding of recombinant proteins. However the major limitation of the dilution method is the problems of scale-up and low yield. Refolding large amount of recombinant protein using dilution method needs large refolding vessel, huge amount of buffer and additional concentration steps after protein renaturation and thus adds to high cost of protein production (Panda, 2003; Alibolandi and Mirzahoseini et al., 2011). Pulse renaturation involving addition of small amount of solubilized protein to the renturation buffer at successive time interval helps in reducing the volume of buffer thus improves the overall performances of the refolding process. Pulse renaturation processes have been successfully tried for the recovery of gamma interferon and lysozyme (Singh and Panda,

2005; Wang et al., 2007). Renaturation of bioactive protein with little aggregation has also been achieved by addition of low molecular weight additives (Kolaj et al., 2009). The most commonly used low molecular weight additives have been glycerol, L-arginine, low concentration of (1-2 M) urea or guanidine hydrochloride, and detergents. Among the additives, the positive effect of glycerol, L-Arginine/HCl in reducing aggregation have been demonstrated on various proteins like human gamma interferon (Babu et al., 2000; Wang et al., 2009) and for fibroblast growth factor (Alibolandi and Mirzahoseini et al., 2011).

High throughput refolding of inclusion body solubilized protein at high protein concentration have been reported by using on-column refolding using affinity (Wang et al., 2009; Esfandiar et al., 2010), ion-exchange (Wang et al., 2007), hydrophobic interaction (Wang et al., 2006) or gel filtration chromatography (Wang et al., 2008; Chen and Leong, 2010) and diafiltration (Dasari et al., 2008). Intermolecular interaction leading to aggregation is minimized when the folding molecules are isolated through binding to the support matrix. Simultaneous used of denaturant free buffer and optimization of elution condition leads to the purification of protein in bioactive form. Such chromatographic matrices have been successfully used for the refolding of interferons, interleukins expressed as inclusion bodies in *E. coli*. Simultaneous renaturation and purification of inclusion body protein using nickel-chelating chromatography have also been reported for interferons (Wang et al., 2009). Using similar methodology, reduced lysozyme at very high concentration (9 mg/ml) have been successfully refolded in to bioactive form with almost 100% recovery using immobilized liposome chromatography (Panda et al., 2003). This methodology is highly efficient in reducing protein aggregation and thus provides maximum recovery of the refolded protein. Protein refolding can also be achieved by the use of diafiltration and dialysis using ultrafiltration membranes (Dasari et al., 2008). Huge volumes of soulubilized protein material can be processed using such refolding procedures. It was found that use of chromatography results in higher yield of the refolded protein in comparison to dilution method. As affinity chromatography offer multiple advantages of buffer exchange, protein refolding and separation of pure protein, it provides an ideal method for large scale refolding of inclusion body protein (Wang et al., 2009; Esfandiar et al., 2010).

2. 5. 4 Chromatographic Purification Strategies for Recombinant Proteins

Purification is an important step in the production of recombinant proteins. The characteristics of large scale purification schemes, such as conventional chromatography,

have a significant impact on the final cost of production. It is often more efficient to use one of the available tags to 'fish out' the target protein. The purpose for which the protein will be used determines required degree for its purity and authenticity. To purify intracellularly produced proteins the cells are harvested and lysed, which naturally contributes to the complexity of the protein mixture. The advantage of intracellular versus secreted protein is the volume to be handled, i.e., the secreted proteins usually exist diluted in the culture medium. Chromatographic methods (affinity, ion exchange, size exclusion and hydrophobic interaction) can be utilized either in traditional, low pressure or high performance liquid chromatography instrumentation.

2.5.5 Affinity Chromatography

Affinity chromatography together with recombinant DNA technology offers a simple and fast technique to purify proteins to high purity with a single purification step (Tan et al., 2010). Fusion can be made on either side or both sides of the target gene depending on specific application, but the majority of fusion proteins place the tag at the N-terminus of the protein (Nilsson et al., 1997). Although affinity chromatography can be used for laboratory scale purification, its utilization on a preparative scale can represent a major cost for the final protein product. Successful separation by affinity chromatography requires that a biospecific ligand is available, and that it is covalently attached to a chromatographic bed material. Due to the specificity of this recognition, it is often possible to obtain high purity of a protein sample (Arnou et al., 2006; Young et al., 2012). The packing material used, called the affinity matrix, must be inert and easily modified. Agarose is the most common substance used as a matrix, in spite of its relative high costs. The ligands, or "affinity tails", that are inserted into the matrix can be genetically engineered to possess a specific affinity. In a process, the desired molecules adsorb to the ligands on the matrix until desorption is carried out e.g., with a high salt concentration, a competition reaction (e.g., imidazole), strong chelating agents and/or low pH. To date, a large number of different fusion partners that range in size from one amino acid to whole proteins capable of selective interaction with ligand immobilized onto a chromatography matrix, have been described (Nilsson et al., 1997: Young et al., 2012). Immobilized metal affinity chromatography (IMAC) systems have three basic components: an electron donor group, a solid support and a metal ion. The metal ion (usually Ni^{2+}, Co^{2+}, Cu^{2+} or Zn^{2+}) is restrained in a coordination complex where it still retains

significant affinity towards macromolecules (Wang et al., 2009). The use of polyhistidine tags has been demonstrated in a wide range of host cells including *E. coli*, *S. cerevisiae*, insect cells as well as in mammalian cells.

2.5.6 Ion Exchange Chromatography

Ion exchange chromatography (IEC) has been the most widely used technique for the isolation and purification of biological macro-molecules. IEC is able to separate almost any type of charged molecules, from large proteins to small nucleotides and amino acids (Ahamed et al., 2007). The ion exchanger is insoluble solid matrices containing fixed ionogenic groups which bind reversibly to sample molecules (proteins, etc.). Desorption is then brought about by increasing the salt concentration or by altering the pH of the mobile phase. The two major classes of ion exhangers are cation exchangers and anion exchangers, having negatively and positively charged functional groups, respectively. Ion exchange containing sulpho propyl (SP) or diethyl aminoethyl (DEAE) is most frequently used. The major property which govern the adsorption to an ion exchanger is the net surface charge of the protein. Since surface charge is the result of weak acidic and basic groups on proteins, separation is highly pH-dependent. The optimum pH range for IEC for many proteins is within 1 pH units of the isoelectric point. Elution with increasing salt concentration or increasing/decreasing pH of buffer is most commonly used in the IEC (Khalilzadeh et al., 2008; Bell et al., 2009). The higher the net charge of the protein, the higher the ionic strength needed to bring about desorption. Thus, to optimize selectivity in ion exchange chromatography, the pH of the running buffer is chosen so that sufficiently large net charge differences among the sample components are created.

2.5.7 Gel Filtration or Size Exclusion Chromatography

The principle of gel permeation chromatography (GPC; size exclusion or gel filtration chromatography) is based on molecular mass (Wang et al., 2008). Large molecules are excluded from the matrix, whereas intermediate size molecule can partly enter and only small molecules can freely enter the matrix. The porous three dimensional matrix acts as a steric barrier to solute molecules as they attempt to equilibrate with liquid inside and outside the bead. While purifying the protein GPC can also be used to estimate approximate molecular weights. The choice of the appropriate column type depends on the molecular size and physical properties of the proteins to be separated.

2.5.8 Hydrophobic Interaction Chromatography

Hydrophobic Interaction Chromatography (HIC) is used to separate proteins on the basis of relative hydrophobicity. At intermediate ionic strengths (1-1.5 M), proteins may be adsorbed from solution onto hydrophobic surfaces. This adsorption is reversible, and elution is achieved by simply lowering the ionic strength. Consequently, HIC is particularly useful for the purification from high ionic strength biological extracts since binding is performed in the presence of salt, and elution in the absence of salt. The technique may be applied to the purification of most soluble proteins. HIC has now used in laboratory scale, as well as in large scale purification of biomolecules, like serum proteins, nuclear proteins, hormones, recombinant proteins and enzymes (Roettger and Ladisch, 1989; Wang et al., 2004; Bhuvanesh et al., 2010).

2.6 *FLAVIVIRIDAE*

Recent past has seen a global resurgence of viral diseases such as dengue, Japanese encephalitis, and yellow fever. The viruses composing the family *Flaviviridae* are single stranded RNA viruses with positive polarity divided into three genera: *Hepacivirus*, *Pestivirus* and *Flavivirus*. More than half of the known flaviviruses are associated with human disease, including the important human pathogens dengue virus (DENV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), West Nile virus (WNV), and the Tick-borne encephalitis viruses (TBEV) (Vasilakis and Weaver, 2008). The most common outcome of human flavivirus infection is subclinical; however, clinical infections present as a flu-like disease with fever, arthralgia, myalgia, retro-orbital headache, maculopapular rash, leucopenia, vascular leakage, hepatitis and/or encephalitis. Viruses are taxonomically grouped within the *Flavivirus genus* by their antigenic relationships, which correlate with their vector: tick-borne, mosquito-borne, and no known vector. Viruses vectored by *Culex* mosquitoes are generally encephalitic and include the JEV serogroup, where birds are the natural reservoir, while viruses vectored by *Aedes* mosquitoes are viscerotropic and include the DENV serogroup as well as YFV, where humans or primates serve as the natural reservoir (Gaunt et al., 2001).

2.6.1 Flavivirus Genome and Proteins

The flavivirus RNA genome is single-stranded and approximately 11 kilobases in length. The genomic RNA is infectious and therefore of positive polarity. Genomic length

RNA are the only viral mRNAs present in flavivirus infected cells. The genomic RNA has a type I cap at the 5' end (m7GpppAmp) and lacks a poly(A) tail on the 3' end. A striking feature of the flavivirus genome is the presence of a single long open reading frame (ORF) that spans nearly the whole genome, encoding at least 10 proteins (Figure 2.4). The long ORF is flanked on each end by 5' and 3' non- 4 coding regions (NCR), respectively (Chambers et al., 1990; Rice, 1990). The structural proteins capsid (C), membrane (M; expressed in the precursor form prM), and envelope (E) are encoded on the 5' quarter of the genome while the non-structural (NS) proteins compose the remainder (Rice et al., 1986). The ORF generates a single polyprotein in the order 5'-C-prM/M-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'. Processing of the flavivirus polyprotein results in the formation of 10 direct or indirect membrane-associated viral proteins: three structural proteins, seven non-structural proteins, and two polypeptide cleavage fragments. The functions of all ten viral proteins are mentioned in table 2.2.

The capsid (C) protein is a small protein, approximately 120 amino acids long, which constitutes the protein component of the nucleocapsid. The C protein is quite basic, probably acting to neutralize the negatively charged viral RNA in such a compact structure (Rice et al., 1986). The prM protein is the precursor glycoprotein to the structural protein M and undergoes a delayed cleavage to form the M protein, a process that is linked to viral budding/maturation to prevent immature virions with host cell membranes (Li et al., 2008).

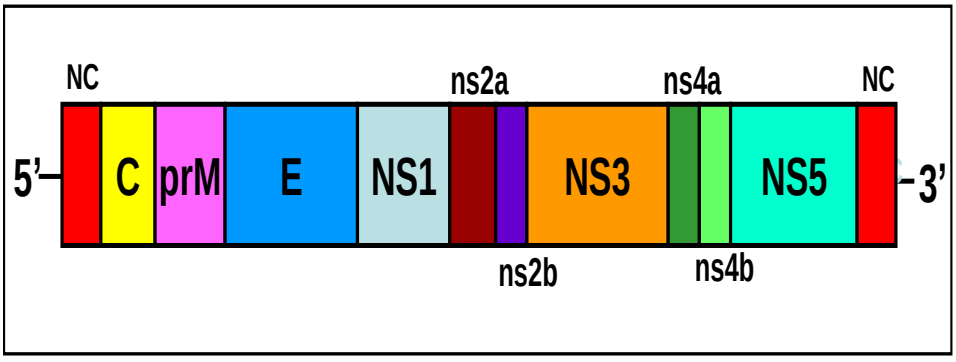

Figure 2.4 Flavivirus genome organization. 5'UTR is followed by the open reading frame coding for the structural and non-structural proteins.

Table 2.2 Flavivirus Proteins.

Protein	Size (kDa)	Number of amino acids	functions
Core	13-16	113	Core protein
Pre-Membrane	19-23	166	Chaperone protein for E; precursor to M
Membrane	8-8.5	75	Component of viral envelope
Envelope	51-60	495	Major envelope protein
NS1	44-49	350-410	Putative RNA replication cofactor
NS2a	16-21	150-210	Putative RNA replication cofactor. Coordinates the shift between RNA packaging and RNA replication
NS2b	12-15	130	Cofactor for serine protease in viral protease complex
NS3	67-76	615	Viral protease; RNA cofactor; NTPase and putative helicase
NS4a	24-32	150-280	Putative RNA replication cofactor
NS4b	10-11	110-250	Innate immunity
NS5	91-98	900	Viral RNA dependent RNA polymerase

The envelope protein is the major surface glycoprotein and is glycosylated in some, but not all flaviviruses. Through crystallographic analysis, each flavivirus E protein has been shown to be composed of three structural domains (Rey et al., 1995) labeled E domain I (EDI) to EDIII. EDI is the central beta-barrel domain, composed of 120 residues in three segments (1-51, 137-189, and 285-302). EDI acts as a flexible hinge region that is important in fusion. This domain is stabilized by two disulfide bridges, and has a conserved glycosylation site at Asn153, with an additional site at Asn 67 in EDII of dengue virus. These glycosylation sites do not have an effect on the antigenic recognition of the E protein by monoclonal antibodies (Heinz and Stiasny, 2012). EDII is the dimerization domain

composed of two segments (52-136, 190-284), and containing three disulfide bridges. EDII also contains an internal type II fusion loop at the distal end, which is packed against EDIII of the adjacent E protein, and undergoes a dramatic acid-mediated conformational change (Guirakhoo et al., 1989).

EDIII has an immunoglobulin (Ig)-like fold and is composed of the C terminal portion of the E protein (303-395). EDIII is joined to EDI through 15 residues and anchored by one intra-monomer disulfide bridge. This interface is partly hydrophobic and partly polar. The Ig beta-barrel lies perpendicular to the virion surface and projects the furthest (Rey et al., 1995). Flavivirus E is involved in several important biological functions. Flaviviruses mediate host cell entry through receptor mediated endocytosis, utilizing EDIII for interaction with cellular receptors (Huerta et al., 2008; Guzman et al., 2010; Pulmanausahakul; 2010). Murine derived monoclonal antibodies (MAb) targeting EDIII, efficiently neutralize virus by blocking viral attachment (Crill and Roehrig, 2001; Heinz and Stiasny, 2012). In addition, recombinant EDIII is able to bind vertebrate target cells through interactions with heparan sulfates and inhibit viral infection (Chu et al., 2005; Hung et al., 2004; Guzmzn et al., 2010; Heinz and Stiasny, 2012).

The NS1 protein contains two or three N-linked glycosylation sites and 12 conserved cysteines, all of which form disulfide bonds. Rapidly after synthesis, NS1 forms highly stable homodimers with a high affinity for membranes; however, due to the largely hydrophilic amino acid content and lack of a transmembrane domain, the nature of this membrane association is unclear. NS1 is largely retained within infected cells (Lindenbach and Rice, 2003) and plays an important, yet unclear role in RNA replication. Anti-NS1 antibodies confer partial protection from virus challenge (Gould et al., 1986; Das et al., 2009; Pulmanausahakul; 2010).

NS2A is a relatively small, hydrophobic, multifunctional, membrane-associated protein involved in RNA replication (Mackenzie et al., 1998). NS2B is also a small membrane-associated protein and forms a stable complex with NS3 and acts as a cofactor for catalytic activity of the viral serine protease. NS3 is a large, multifunctional protein required for polyprotein processing and RNA replication. NS4A/2K is a small hydrophobic protein that localizes to sites of RNA replication and interacts with NS1, which is essential for RNA replication, suggesting a role in viral replication (Mackenzie et al., 1998). NS4B is the

largest of the small hydrophobic NS proteins, consisting of 248 amino acids. Coexpression of NS4A and NS4B enhances the inhibition of interferon-stimulated response element promoter activation in response to IFNα/β stimulation (Munoz-Jordan et al., 2005). NS5 is the largest and most conserved of the flavivirus proteins, composed of approximately 900 amino acids. NS5 is essential in replication containing domains for RNA-dependent RNA polymerase (RdRp) and 5' capping.

2.7 THE DENGUE VIRUS

Dengue virus serocomplex is composed of four distinct species within the flavivirus genus, which includes four genetically different, yet antigenically related serotypes (Dengue-1, -2, -3, and -4) (Calisher et al., 1989). All four serotypes circulate in urban environments of the tropics and subtropics between humans and the peridomestic *Aedes aegypti* mosquito. This distribution puts nearly a third of the global population at risk for dengue virus infection (Farrar et al., 2007; Whitehorns and Simmons, 2011). Dengue virus infection was reported in the 1780 Philadelphia outbreak, it was probably rare and did not pose a serious public health problem (Vasilakis and Weaver, 2008). The first well documented cases of DHF were associated with epidemics in Thailand and the Philippines in the 1950s and thought to be a new disease. Since this time DHF/DSS have become endemic in all countries in Southeast Asia, with dramatic increases in case numbers, so much so that dengue is considered an archetypal "emerging" disease. The most common clinical disease caused by dengue infection is dengue fever (DF), a febrile illness of older children and adults. DF is commonly associated with 5-7 day duration of fever, headache, retroocular pain, myalgia, arthralgia, and frequently a generalized maculopapular rash on the trunk. Treatment for DF is supportive and patients normally fully recover. Severe manifestations of dengue virus infection include dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Children under 16 are at the greatest risk for developing DHF/DSS. Severe disease and shock is frequently observed the day following defervescence and rarely lasts more than 48 hours. DHF/DSS is characterized by fulminant hepatitis, massive hemorrhage including positive tourniquet test, petechia, intestinal hemorrhage, and hemetemesis, organ failure, vascular leak with hypovolemic shock induced by plasma leakage, thrombocytopenia of less than 100,000 cells per mm^3, elevated liver enzymes, and coagulation disorders including decreased fibrin and increased fibrin degradation products. Currently dengue has caused more illness and death

than any other arboviral diseases of humans, with an estimated 100 million cases of dengue fever and 500,000 DHF cases occurring annually in the world. The case fatality varies from 0.5 to 3.5%, depending on severity of epidemic and is the highest in Asian countries. DHF is the leading cause of hospitalisation and death among children in Southeast Asian countries, where more than 90% of the DHF patients are children below the age group of 15 years. In the last two decades, outbreaks of dengue infections have accelerated enormously in India, with largest and severe being the DHF epidemic in Delhi in 1996, indicating a serious resurgence of dengue virus infection (Chaturvedi and Nagar, 2008; Dash et al., 2011). The geographic distribution of dengue is shown in figure 2.5.

Figure 2.5 Geographic distribution of dengue virus endemic areas in 2011 (WHO, 2012).

Developing a vaccine for dengue virus is a very challenging task because of few complications (Guy et al., 2011; Thomas and Endy, 2011). Dengue virus has four serotypes, infection with one-serotype results in lifelong immunity to it but there is no cross-protection against the others. Persons living in the areas of endemicity can be infected with any one of the other three serotypes causing clinical illness, ranging from a non-specific febrile syndrome to severe and fatal dengue hemorrhagic fever (DHF) /dengue shock syndrome (DSS). Protection against any one or two of the dengue virus serotypes can increase the risk of more serious disease through the antibody dependent enhancement (ADE) phenomenon. During the secondary infection with heterologous dengue serotype, antibodies could

recognize the viruses and bind to them forming dengue virus-antibody complexes. These complexes are taken up more readily than uncoated viruses by mononuclear cells bearing Fcγ receptors, especially macrophages. However, these cross-reactive antibodies fail to neutralize the viruses, so the viruses could replicate freely inside the cells. This phenomenon is called antibody-dependent enhancement (ADE) (Pulmanausahakul et al., 2010; Guzman et al., 2010; Simmons et al., 2012).

2.7.1 Dengue Diagnosis

The diagnosis of dengue from clinical symptoms is not reliable, as around 50% of dengue patients will have only mild, undifferentiated fever. Symptomatically, dengue resembles Chikungunya fever, Leptospirosis, Typhoid and Malaria. So confirmatory diagnosis can only be achieved with laboratory support. The current trends of routine laboratory diagnosis include virus isolation, detection of antibody or antigen by serodiagnosis and/or molecular detection by demonstration of viral RNA by RT-PCR, Real time PCR (Guzman and Kouri, 1996; Peeling et al., 2010; Tripathi et al., 2010). Virus isolation is the gold standard routinely followed in the clinical laboratory. But it is a difficult task since dengue virus is highly fastidious and slow growing. For virus isolation, the sample should preferably be collected within 5-6 days of fever (viremic acute phase patient sera) (Yamada et al., 2002). Post-viremic sera are most likely to be dengue positive by serological tests, which are often complicated and time consuming. These techniques are labour intensive, expensive and cumbersome. Thus, in a majority of cases the only feasible diagnostic test would have to be based on the identification of anti-dengue IgG and IgM antibodies. ELISA or immunochromatography test is the most widely used serological test to diagnose dengue infection. Diagnostic kits based on whole virus are expensive due to the high costs associated with antigen production (Hapugoda et al., 2007; Tan and Ng, 2010). Now a days recombinant protein based ELISA for detection of antibodies may replace the native antigen based assays. The test is simple, rapid, sensitive and is also capable of discriminating between primary and secondary infections by detecting IgM and IgG antibodies (Hapugoda et al., 2007; Tripathi et al., 2011). Thus the aim of the present work is to evaluate recombinant dengue virus envelope domain III protein for detection of anti dengue IgM and IgG antibodies to detect dengue infection.

2.7.2 Dengue Vaccines

The development of a vaccine against dengue has been a high priority of the World Health Organization (WHO) for decades. Inactivated viral vaccines for other closely related flaviviruses, such as yellow fever, Japanese encephalitis and tick borne encephalitis viruses, are available but no safe and effective vaccine or therapeutic measures has been developed for dengue virus till date. The current status of flaviviral vaccines development is shown in table 2.3 (Pulmanausahakul et al., 2010; Simmons et al., 2012). Currently many virus-based dengue vaccines are in various stages of development (Table 2.3). Two of these are traditional tissue culture-based live attenuated vaccines whereas the remaining four are chimeric recombinant vaccine viruses developed using infectious clone technology. In all these instances, the vaccine viruses are monovalent in that each one is specific to one dengue serotype. A tetravalent dengue vaccine is based on producing vaccine formulations by mixing all four monovalent vaccine viruses. Investigations using virus-based recombinant and non-recombinant dengue vaccine candidates in monkeys (Guirakhoo et al., 2002) and man (Sabchareon et al., 2002), respectively, have shown that the current strategy of creating tetravalent dengue vaccine formulations can lead to an unbalanced immune response, specific to only one particular serotype. While efforts are underway to optimize the tetravalent formulations, the inherent risk of viral interference associated with the current strategy of producing tetravalent dengue vaccine warrants investigation of other vaccines that eliminate this risk of viral interference. The primary drawback is that the attenuated virus may revert back to its virulent state while living in the host's body. This formalin-inactivated type of vaccine is probably the most common on the market today; however, despite the widespread use of this technique of vaccine preparation the resulting products are really not very effective because the formalin treatment often chemically alters the antigen in some way so as to render it less potent in eliciting an immune response in the host. Where as DNA based vaccines tend to induce relatively low antibody titres, presumably due to the poor efficiency of target cell transduction and concomitant low-level antigen expression. Induction of low titres of antibodies is potentially risky in the context of ADE.

Recent developments and success in recombinant subunit protein vaccines for several viral diseases like hepatitis, malaria and anthrax opened new opportunities in dengue vaccine research. Keeping in view of the present scenario of severity and spread of dengue fever, studies on evaluation of new candidate recombinant dengue antigens for possible

Table 2.3. Current flaviviral vaccines (Pulmanausahakul et al., 2010; Simmons et al., 2012).

Pathogen	Available vaccines	Vaccines under development
Yellow fever	YF 17D live attenuated virus	None
Japanese encephalitis	Inactivated mouse brain derived; Inactivated cell culture derived; SA 14-14-2 live attenuated	ChimeriVax-JE; IC 51; Inactivated cell culture derived; Adenovirus type 5 vectored prM-E virus like particles subunit and naked DNA
Dengue types 1-4	None	Live attenuated yellow fever 17D/DENV chimeric vaccine; PDK cell-passaged, live attenuated vaccine; Live attenuated DENV Delta-30 mutation and intertypic DENV chimeric vaccines; Dengue prM-E DNA vaccine; Recombinant 80% E subunit antigen vaccine; Purified inactivated vaccine (PIV); Live attenuated chimeric DENV vaccine
West Nile	No human vaccines; Veterinary inactivated cell culture derived vaccines	Inactivated cell culture derived; ChimeriVax-WN; DEN-4/WN chimera
Tick-borne encephalitis	Inactivated cell culture derived	Recombinant live DEN-4/TBE or DEN-4/Langat chimeras Virus attenuated by C protein deletion prM-E virus-like particles truncated E protein subunit infectious DNA/RNA encoding attenuated/nonreplicating vaccines
Kyasanur forest disease	Inactivated cell culture derived	None

vaccine development is the need of the hour. Since protein subunit vaccines employ only a portion of a virus, they are easy, safer in production and use than live vaccines containing the whole genome. Moreover production of subunit vaccines involves low cost when compared to inactivated vaccines. Recombinant DNA techniques provided the possibility of cloning specific genes encoding for protective antigens and of expressing them in other host cells, including *E.coli*, Yeast and insect cell systems. This technology has been used by several researchers for the development of subunit vaccines.

Majority of efforts have been made to express the recombinant envelope protein (Kelly et al., 2000; Jaiswal et al., 2004; Pattnaik et al., 2006; Zhang et al., 2007; Batra et al., 2010; Pulmanausahakul et al., 2010; Guzman et al., 2010; Valdes et al., 2011; Clements et al., 2010; Coller et al., 2011; Chiang et al., 2012; Yang et al., 2012) while few have tried to express non structural proteins (Das et al., 2009; Pulmanausahakul et al., 2010; Simmons et al., 2012; Amorim et al., 2012) subunit vaccine candidates.

2.7.3 Dengue Virus Envelope Domain III Protein as Diagnostic and Vaccine Candidate

The envelope protein comprises 3 regions: Domain I, Domain II and Domain III (Figure 2.6). Experimental evidences have shown that the EDIII protein is a receptor binding domain (Chin et al., 2007; Zhang et al., 2007; Block et al., 2010; Coller et al., 2011; Simmons et al., 2012). In addition, it has also been demonstrated to be highly immunogenic and able to elicit the production of neutralizing antibodies against the wild-type virus (Guzman et al., 2010; Tan et al., 2010; Chiang et al., 2012). The binding of the DIII-specific neutralizing antibodies to the virus caused an alteration of the spatial arrangement between the glycans on the E proteins. These changes in the structure of the viral surface were presumably responsible for inhibiting attachment to the cells (Lok et al., 2008). One major challenge to dengue virus vaccine development is the potential development of antibody-dependent enhancement (ADE) of virus replication, which is believed to cause DHF and DSS (Simmons et al., 2012). In addition, immunization against one dengue serotype induces life-long immunity against the homologous serotype and short-lived immunity against the other serotypes. Put together, it is widely believed that for a dengue virus vaccine to be effective, it must comprise neutralizing epitopes from all four serotypes (tetravalent) (Tan and Ng, 2010). Presently, EDIII protein immunization in animal has demonstrated promising results. In these

studies, a variety of parameters affecting recombinant dengue virus EDIII protein immunogenicity has been investigated. These parameters include: antigen combination - monovalent, bivalent, or tetravalent EDIII and type of animal model used (Table 2.4).

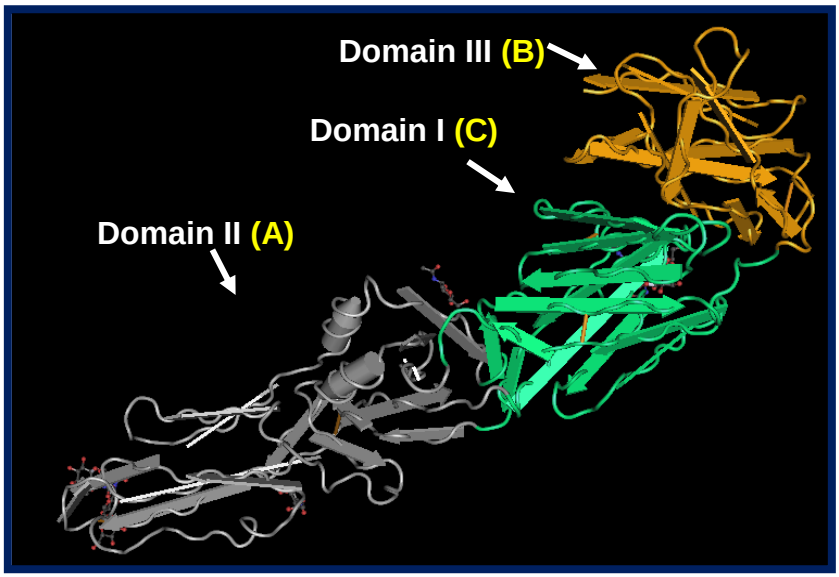

Figure 2.6. Structural organization of dengue virus envelope protein-domain I, II and III.

On the whole, recombinant EDIII protein immunization generated satisfactory levels of antibodies that are neutralizing against the virus. This observation is consistent for dengue virus and West Nile virus EDIII proteins expressed from *E. coli* (Babu et al., 2008; Chin et al., 2007; Martina et al., 2008). Unlike live attenuated vaccines, protein subunit vaccines need to be adjuvanted in order to elicit a suitably good immune response (Whitehead et al., 2007; Chiang et al., 2012). It has been observed that recombinant EDIII protein mixed with either complete/incomplete Freund's Adjuvant, PELC, CpG or Montanide ISA 720 adjuvants elicited polyclonal antibodies (in mice) with higher neutralizing efficacies ($PRNT_{90}$ of 1:128) as compared to the neutralizing antibodies generated using an recombinant EDIII protein-Alum mix ($PRNT_{90}$ of 1:64). Cell-mediated immune responses were also varied according to the type of adjuvant used (Babu et al., 2008; Chiang et al., 2012). However, the true potential of envelope domain III as a potential vaccine candidate has not been thoroughly investigated. Nonetheless, generation of recombinant dengue virus EDIII protein from infected tissue culture insect cells is a laborious and costly, subjected to batch-to-batch variation making it difficult to routine large-scale production. Production of recombinant EDIII protein in

Table 2.4. Recent studies on recombinant dengue virus EDIII based vaccine.

Protein(s) tested	Animal model	Results	Authors and years
rDen 2 EDIII	Rat	Generation of anti-dengue 2 EDIII antibodies	Saejung et al., 2006
rDen 1 and rDen 2 EDIII	Balb/C mice	Generation of neutralizing antibodies invitro and blocking of viral entry	Chin et al., 2007
Tetravalent rDen 1-4 EDIII	Balb/C mice	Protection against dengue virus 1, 2 and 4 (80%) and dengue virus 3 (18%)	Chen at al., 2007
Tetravalent rDen 1-4 EDIII (Expressed in *Pichia pastoris*)	Balb/C mice	Generation of neutralizing antibodies against all four dengue serotypes	Etemad et al., 2008
rDen 2 EDIII	*Macaca fascicularis* monkeys	Generation of neutralizing antibodies against dengue virus 2	Bernardo et al., 2008
Tetravalent rDen 1-4 EDIII	Balb/C mice	Generation of un-balanced neutralizing antibodies against dengue virys 1-4	Block et al., 2010
rDen 1 EDIII	Balb/C mice	Generation of neutralizing antibodies against dengue virus 1	Tan et al., 2010
rDen 2 EDIII	BALB/c and C57BL/6 mice	Generation of neutralizing antibodies against dengue virus 2	Yang et al., 2012
rDen 1 EDIII	Balb/C mice	Generation of neutralizing antibodies against dengue virus 2	Chiang et al., 2012

E.coli strains is a much cheaper and a simpler procedure (Jaiswal et al., 2004; Zhang et al., 2007; Tripathi et al., 2008; Tan et al., 2010; Tripathi et al., 2011). Currently, there is a need for the production of cost effective and safe EDIII protein related biologics for the development of protein subunit vaccines or diagnostic reagents. For these purposes, the recombinant proteins produced must maintain their biological activity (i.e., generate neutralizing antibodies against wild-type virus or able to bind to anti-dengue virus antibodies found in patient serum).

Currently, dengue diagnosis is based on serology, virus isolation and RNA detection (Tripathi et al., 2011). Because of its sensitivity and ease of use, the ELISA platform is widely used as surveillance tool to detect antigen or anti dengue IgM and IgG antibodies in patient serum samples. Although to date, there is no EDIII protein-based diagnostic assay available commercially, many in-house tests have demonstrated the possibility of using the EDIII protein as a reagent to serologically detect dengue infection (Jaiswal et al., 2004; Hapugoda et al., 2007; Tripathi et al., 2008, 2009; Batra et al., 2011). IgM and IgG ELISA performed using recombinant dengue virus EDIII protein coated immuno-plate was used for the detection of antibodies in patient serum samples (Hapugoda et al., 2007; Batra et al., 2010, 2011). IgG-ELISA was similarly performed using EDIII antigens for detection of anti-EDIII antibodies in mice serum (Khanam et al., 2007; Babu et al., 2008; Coller et al., 2011). In addition, tetravalent EDIII proteins could also be used for the detection of antibodies against dengue virus via an IgG-ELISA platform (Chen et al., 2007; Etemad et al., 2008). Based on these findings, the EDIII protein demonstrates great potential in being produced as diagnostic reagents for the development of serological tools such as ELISA. These tools, if validated and available commercially, can enhance mass screening of patient serum samples and allow rapid initial detection of dengue virus suspect cases in clinics. The dengue EDIII protein is highly immunogenic and has vast potential as a protein subunit vaccine as well as a diagnostic reagent for dengue diagnosis. The current work is based on the premise that the development of a diagnostic and vaccine based on the production and purification of envelope domain III of dengue virus serotypes and the detailed study on their diagnostic potential as well as immunogenicity in mice models. Further studies are required to increase the Immunogenicity of subunit vaccines by incorporating them into adjuvants or other systems for stimulating immune responses.

2.8 THE JAPANESE ENCEPHALITIS VIRUS

One of the most important viral encephalitis causative agents in Asia is Japanese encephalitis virus (JEV) which is transmitted to human by *Culex* mosquitoes, principally *Culex tritaeniorhynchus* and *Culex quinquefasciatus*, which have fed on viremic animals, mostly domestic pigs. The geographic distribution (Figure 2.7) of JEV is mainly in the Asian region including India, China, and all of South-East Asia, although outbreaks have also been reported in Pakistan, Nepal and Australia. With nearly 3 billion people living under the current JE-endemic region, recurring incidents of epidemic are being reported at regular intervals. From the 1870s, recurring epidemics of encephalitis have been reported from the islands of Japan, especially during the summer season, with major outbreaks occurring every 10 years. In 1935 the Nakayama strain of Japanese encephalitis virus (JEV) was isolated from the brain of a fatal case. Since then, the virus has spread in South Asia, Southeast Asia

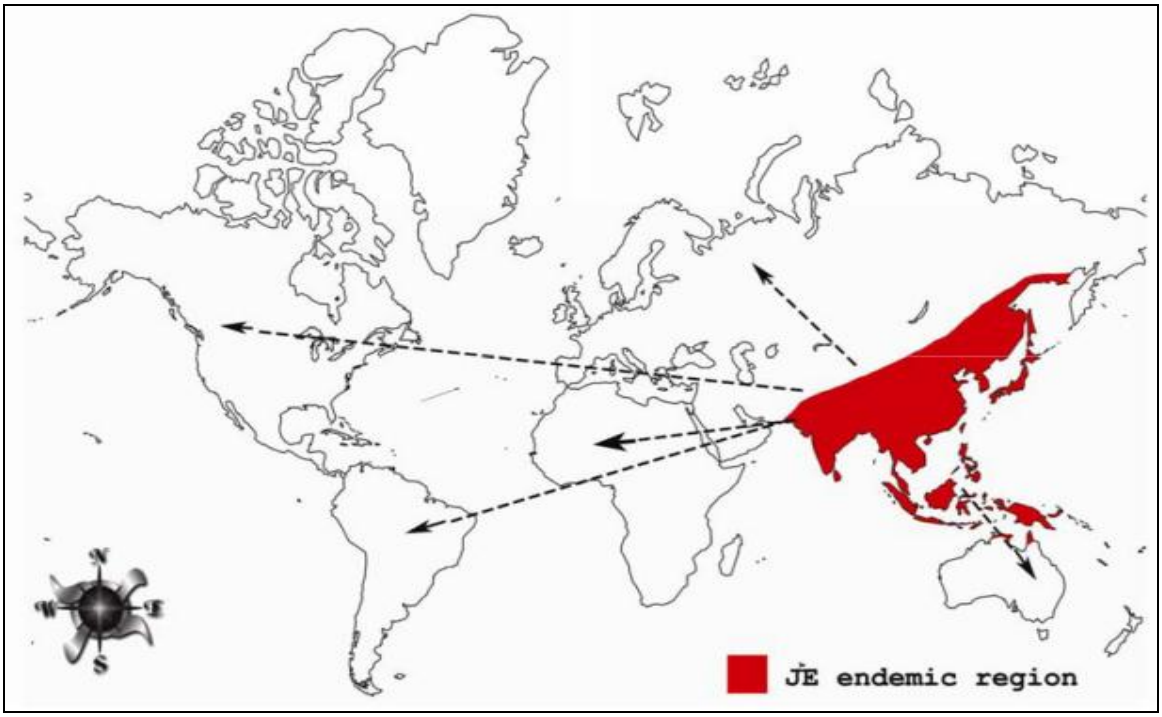

Figure 2.7 Geographical distribution of Japanese encephalitis (Ghosh and Basu, 2009).

and the Pacific regions. Rapid globalization, population explosion, changes in global climatic condition, industrialization and deforestation, all seem to correlate with the spread of the virus into newer territories (Ghosh and Basu, 2009). In India, JE as a disease was first reported in 1955 when clinical cases were detected in Vellore and Pondicherry in southern

India. From then onwards to till date there have been JE cases reported JE in India every year (Dutta et al., 2010; Pujhari et al., 2011). In endemic areas, at least 50,000 cases of Japanese encephalitis (JE) are reported annually, among which some 10,000 cases result in death. Because of the high mortality rate, the disease is considered as one of the most important health problems in the South-East Asia region. A typical illness starts with fever and other symptoms including vomiting and photophobia. Neurological symptoms develop subsequently, with a spectrum ranging from neck stiffness, stupor and impaired consciousness to seizures, parkinsonian movement disorders, convulsions, flaccid paralysis and coma. Gradually the fever rises and encephalitis starts at which point the fatality rate is about 35% (Dutta et al., 2010; Pulmanausahakul et al., 2010; Abraham et al., 2011). Due to lack of effective medical treatment or a specific antiviral, attempts to develop a cost effective diagnostic and vaccine candidate against the JE are considered a high priority.

2.8.1 Japanese Encephalitis Diagnosis

Due to lack of definitive therapeutic for JE, early diagnosis plays major role in patient management. The clinical picture of JE resembles the picture of acute encephalitis syndromes of other aetiologies and cannot be differentiated accurately. The confirmatory diagnosis therefore requires antigen or antibody determination. The routine laboratory diagnosis of JEV infection is based on virus isolation and serology followed by identification of the viral genome by reverse transcription polymerase chain reaction (RT-PCR) or real time PCR. PCR is based on the identification of the virus or its RNA genome which persists in circulation during short period of viremia (Parida et al., 2006; Ravi et al., 2006; Santhosh et al., 2007; Li et al., 2012). Virus isolation and PCR is costly and requires sophisticated setup. Cultivation of JEV from blood and cerebrospinal fluid (CSF) is rarely positive as the viraemic period lasts only a few days, thus diagnostics rely mostly on serological assays. The flavivirus group shows intense cross-reactivity which is highest on the IgG level. However IgM is relatively specific for the infecting virus. IgM is detectable in CSF and blood from almost all patients within 7 days after onset of disease. Since most of the JE cases occurs in rural areas, IgM ELISAs are used most commonly (Jacobson et al., 2007; Ghosh and Basu, 2009; Ravi et al., 2009; Litza et al., 2010; Robbinson et al., 2010). Diagnostic kits based on whole virus are expensive due to the high costs associated with antigen production (Shrivastva et al., 2008). Now a days recombinant protein based ELISA for detection of

antibodies may replace the native antigen based assays (Tan and Ng, 2010; Chavej et al., 2010). Thus, in a majority of cases the only feasible diagnostic test would have to be based on the identification of ant-JEV IgM antibodies by ELISA. Thus the aim of the present work is to evaluate recombinant JEV envelope domain III protein and nonstructural 1 protein in ELISA for detection of anti JEV IgM antibodies to detect JEV infection

2.8.2 Japanese Encephalitis Vaccine

Due to lack of definitive therapeutic countermeasures to combat JE, implementation of prophylactic strategies remains the best way to prevent JE. Human vaccination remains, till date, the most effective measure to prevent JE. Multiple vaccines exist to control JE (Table 2.3.), but all have their limitations. The available vaccines for JE is based on the mouse brain or cell culture derived formalin inactivated vaccine. Immunization against the virus is the single most important control strategy that could reduce dramatically the numbers of JE cases (Nitatpattana et al, 2008; Halstead and Thomas, 2010). Purified formalin-inactivated mouse brain-derived vaccine and an inactivated Vero cell culture-derived vaccine have been licensed in the United States and a live attenuated vaccine (SA 14-14-2) is used in many countries in Asia (Misra and Kalita, 2010). These 3 commercially available vaccines against JE are based on the Nakayama, Beijing-1, and SA14-14-2 strains, which belong to genotype III (Dutta et al., 2010; Abraham et al., 2011; Liu et al., 2011). Despite widespread availability of these vaccines, it is comparatively expensive for large scale use in many Asian countries and there have been reports of severe allergic reactions to this vaccine (Plesner and Ronne, 1997; Pulmanausahakul et al., 2010). Considering cost and safety, recombinant protein based JEV vaccine is necessary for human use.

Several efforts have been and are still being made to develop recombinant vaccines for JEV. Amongst the JEV viral proteins, JEV E protein seems to be the most suitable one for plasmid DNA based vaccines, since antibodies against E protein are capable of neutralizing JEV activity. Plasmid constructs that contain JEV E protein together with the prM protein have been shown to provide a protective immune response to lethal JEV challenge in adult mice (Chang et al., 2000; Konishi et al., 1999). There are also DNA vaccines candidates utilizing non-structural proteins of JEV. Immunization of mice with plasmid DNA constructs containing JEV NS1 provided 90% protection against lethal challenge with JEV while plasmids being longer constructs failed to provide protection.

However, vaccines utilizing JEV NS5 or NS3 failed to raise an effective immune response (Liang et al., 2009). Recombinant virus based vaccines have also been reported in JEV vaccine development. JEV structural and non-structural proteins including prM, E, NS1 and NS 2B expressed in different systems can produce a protective immune response in mice (Lin et al., 2008; Wang et al., 2009; Witthajitsomboon et al., 2010; Pulmanausahakul et al., 2010; Lin et al., 2012). Envelope domain III based recombinant vaccine against JE virus has been proposed which carry dominant epitopes that induce neutralizing antibodies, has been created. JEV envelope domain III protein has also been evaluated in murine models (Li et al., 2012; Tafuku et al., 2012). Thus, present study targeted the process development for production of envelope domain III protein of JE virus and its characterization for diagnostic and vaccine utility.

2.8.3 JEV EDIII Protein as a Potential Diagnostic and Vaccine Candidate

Inactivated JEV vaccines, prepared from infected mouse brains (BIKEN or JEVAX) or primary hamster kidney cells, have been successfully developed to control JEV infection (Dutta et al., 2010; Halstead and Thomas, 2010). However, a major problem associated with the inactivated mouse brain vaccine is its failure to stimulate long-term immunity. As a result, multiple immunizations are recommended to provide adequate protection. In addition, inactivated mouse brain or cell culture derived whole virus vaccines are costly to prepare and carry the risk of allergic reactions. The World Health Organization has designated JEV vaccines as a high-priority target for further research and development. Several experimental recombinant, attenuated, subunit and DNA-based JEV vaccines have been reported (Pulmanausahakul et al., 2010) however, only limited protective immunity could be obtained. Hence, new strategies should be developed to produce safer and more efficacious vaccines against JEV infection. The envelope protein is formed as a flat, elongated dimer on the virus surface, and each monomer consists of three domains: domain I; domain II and domain III (Figure 2.6). Among these three domains, only the domain III can be independently folded as an individual fragment that requires one disulfide bridge to maintain its conformational structure. Envelope domain III carries several epitopes capable of eliciting virus-neutralizing antibodies (Alka et al., 2003; Verma et al., 2009). Since the envelope domain III protein can fold independently into a stable conformation, it is therefore an attractive target to block infectivity either through the design of molecules that would compete with whole virus to enter cells or by eliciting neutralizing antibodies

(Wu et al., 2003; Lin et al., 2008; Ramanathan et al., 2009). Experimental evidences have shown that the EDIII protein is a receptor binding domain and demonstrated to be highly immunogenic and able to elicit the production of neutralizing antibodies against the JE virus (Alka et al., 2003; Tafaku et al., 2012). Recombinant JEV EDIII protein immunization in animal has demonstrated promising results and generated satisfactory levels of antibodies that are neutralizing against the virus. This observation is consistent for dengue or West Nile virus proteins expressed from *E. coli* (Tan and Ng, 2010). It has been observed that EDIII protein mixed with Adjuvant elicited polyclonal antibodies (in mice) with higher neutralizing efficacies as compared to the neutralizing antibodies generated using an EDIII protein. In addition it should also be useful to identify receptors and co-receptors located at the cell surface for the development of a receptor blocking vaccine (Alka et al., 2003; Verma et al., 2009). Nonetheless, generation of recombinant JEV EDIII protein from infected tissue culture insect cells is a laborious and costly, making it difficult for routine large-scale production. Production of recombinant EDIII protein in *E.coli* strains is a much cheaper and a simpler procedure (Lin et al., 2008; Tafuku et al., 2012).

Currently, JE diagnosis is based on serology, virus isolation and RNA detection (Ghosh and Basu, 2009). Because of its sensitivity and ease of use, the ELISA platform is widely used as surveillance tool to detect antigen or anti JE IgM antibodies in patient serum and CSF samples (Jacobson et al., 2007; Ravi et al., 2009; Litza et al., 2010). Although to date, there is no EDIII protein-based diagnostic assay available commercially, in-house tests have demonstrated the possibility of using the EDIII protein as a reagent to serologically detect JE infection. IgM ELISA performed using recombinant JE virus EDIII protein was used for the detection of antibodies in patient serum and CSF samples (Tripathi et al., 2010). IgG-ELISA was similarly performed using EDIII antigens for detection of anti-EDIII antibodies in mice serum (Verma et al., 2009; Lin et al., 2008; Tafuku et al., 2012). Based on these findings, the EDIII protein demonstrates great potential in being produced as diagnostic reagents for the development of serological tools such as ELISA. These tools, if validated and available commercially, can enhance mass screening of patient serum and CSF samples and allow rapid initial detection of JE virus suspect cases in rural areas. For this reason, the EDIII protein is an important immunogen for the development of a prospective protein subunit vaccine and also a prospective diagnostic reagent for the improved clinical diagnosis of JEV infections. However, the true potential of domain III as a potential vaccine candidate has not been thoroughly investigated. Further studies are required to increase the

Immunogenicity of subunit vaccines by incorporating them into adjuvants or other systems for stimulating immune responses. The objective of the study is to obtain high yield, refolded and purified envelope domain III protein of JEV for potential use in diagnostic and vaccine development studies.

2.8.4 JEV Nonstructural 1 Protein as a Potential Diagnostic and Vaccine Candidate

Although the function of the NS1 is not elucidated fully, available evidence suggests that this protein is involved in viral RNA replication (Abraham et al., 2011). In addition, the high immunogenicity of the NS1 proteins of JE and other flaviviruses has raised considerable interest both as an antigen for diagnostic methods (Konishi et al., 2009; Allonso et al., 2011; Kassim et al., 2011; Li et al., 2012) and as component of subunit vaccine formulations (Lin et al., 2008; Amorim et al., 2010; Li et al., 2012; Tafuku et al., 2012). Attempts to express the dengue virus NS1 protein in *Escherichia coli* strains have obtained limited success due mainly to the insolubility of the recombinant protein. In addition, the lack of post translational modifications and altered secondary structure of the recombinant protein affects the formation of dimers and results in decreased immunogenicity and antigenicity (Das et al., 2009). Nonetheless, generation of recombinant NS1 protein from infected tissue culture insect cells is a laborious and costly, subjected to batch-to-batch variation making it difficult for routine large-scale production. Production of recombinant NS1 protein in *E.coli* strains is a much cheaper and a simpler procedure (Lin et al., 2008; Das et al., 2009; Amorim et al., 2010; Tafuku et al., 2012). Antibodies to JEV nonstructural proteins constitute a marker of natural infection among vaccinated populations. NS1 antigen was used in ELISA to detect antibodies in horse sera (Konishi et al., 2004). An ELISA using JEV NS1 antigen has been reported to measure anti JEV NS1 antibodies induced in sera of vaccinated clinical cases (Konishi et al., 2009). However, the true potential of JEV NS1 antigen as a potential diagnostics or vaccine candidate has not been thoroughly investigated. The present study aims to establish production systems in *E. coli* for high yield of recombinant JEV NS1 protein and an in-house ELISA using this recombinant protein for detection of IgM antibody to JEV NS1 in human sera and CSF samples.

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CHAPTER 3

MATERIALS AND METHODS

The materials and methods described in the following section apply to all experiments. These are general materials and methods which have been used throughout the whole work, although specific contents used in particular parts of it can be also found in the corresponding chapters.

3.1 STRAINS AND VECTORS.

Three different *E. coli* based systems were used to overexpress recombinant JE virus envelope domain III and NS1 protein as well as dengue virus envelope domain III protein. All the three strains were stored at -80ºC in glycerol stocks made from exponential phase culture in Luria Bertani medium supplemented with antibiotics.

The first expression system was used to overexpress recombinant JE virus envelope domain III protein with a hexahistidine tag. The *E. coli* strain BL21 (DE3) [Novagen, USA] harbouring recombinant plasmids pET30a+ (Novagen, USA) encoding envelope domain III gene of JE virus was used for its overexpression. This strain was previously cloned and transformed with a commercially available plasmid, pET30a+ inducible expression vector, in which the rJEV EDIII gene was inserted into the *Nde*I and *Xho*I sites and the resultant *Nde*I-*Xho*I fragment was cloned downstream of T7 promoter of *E. coli* expression vector pET30a+ to yield plasmid pET-JEV EDIII. The resultant transformants were selected on kanamycin plates.

The second expression system was used to overexpress recombinant JE virus nonstructural 1 protein with a hexahistidine tag. The *E. coli* strain SG13009 (Qiagen, Germany) harbouring recombinant plasmids pQE-30UA (Qiagen, Germany) encoding nonstructural 1 gene of JE virus was used for its overexpression. This NS1 gene was previously cloned downstream of T5 promoter of *E. coli* expression vector pQE-30UA to yield plasmid pQE-JEV NS1. The resultant transformants were selected on ampicillin and kanamycin plates.

The third expression system was used to overexpress recombinant dengue virus type 1 to 4 envelope domain III proteins separately with a hexahistidine tag. The *E. coli* strain BLR (DE3) [Novagen, USA] harbouring recombinant plasmids pET30a+ encoding envelope domain III gene of dengue virus serotype 1 to 4 separately was used for overexpression. Transformation of this strain was performed using commercially available plasmid, pET30a+

inducible expression vector, in which recombinant dengue virus type 1, 2, 3 and 4 envelope domain III gene was inserted into the *Nde*I and *Sal*I sites and the resultant *Nde*I-*Sal*I fragment was previously cloned downstream of T7 promoter of *E. coli* expression vector pET30a+ to yield plasmid pET-rDen 1, 2, 3 and 4 envelope domain III gene. The resultant transformants were selected on kanamycin and tetracycline plates.

3.2 CHEMICALS, BIOLOGICALS AND OTHER CONSUMABLES

All chemicals used in this study were purchased from Sigma, USA. All the media and its components were obtained from Difco, USA. SDS-PAGE related reagents were obtained from Bio-Rad, USA. Protein molecular weight markers and silver staining kit were from MBI Fermentas, USA. HRP conjugated anti-mouse IgG antibodies, HRP-conjugated anti-human IgG and IgM antibodies. Antibiotics and IPTG were purchased from Sigma, USA. HRP conjugated anti-his antibodies (6x His) were from Qiagen, Germany. Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) were procured from Sigma, USA. BCA protein assay kit was from Pierce, USA.

3.3 ANTIBIOTICS AND INDUCER

The concentration of antibiotics and inducer used in this study are given in table 4.1.

Table 4.1 Antibiotics and inducer for shake flask culture and fermentations.

Reagent	Stock solution	Final concentration in use
Tetracyclin	12.5 mg/ml	12.5 µg/ml
Kanamycin	50 mg/ml	50 µg/ml
Ampicillin	100 mg/ml	100 µg/ml
IPTG	238 mg/ml	1 µg/ml

3.4 MEDIA COMPOSITION

Detailed media composition used for shake flask culture, batch and fed-batch fermentations are given below. All the media were prepared in triple distilled water (TDW) and sterilized by autoclaving for 20 min at 15 psi pressure unless otherwise indicated.

(a) Luria bertani (LB) broth (per liter)

Tryptone	10 g
Yeast extract	5 g
Sodium chloride	5 g

(b) Super optimal broth (SOB) (per liter)

Tryptone	20 g
Yeast extract	5 g
Sodium chloride	0.5 g
Magnesium sulphate	2.4 g
Potassium chloride	0.186 g

(c) Super optimal broth with catabolic repressor (SOC) (per liter)

Tryptone	20 g
Yeast extract	5 g
Sodium chloride	0.5 g
Magnesium sulphate	2.4 g
Potassium chloride	0.186 g
Glucose	3.6 g

(d) M9 minimal medium (per liter)

Di-sodium phosphate	6.78 g
Mono-potassium phosphate	3 g
Sodium chloride	0.5 g
Ammonium chloride	1 g
Glucose	20 g

(e) Terrific broth (per liter)

Tryptone	12 g
Yeast extract	24 g
Di-potassium phosphate	9.4 g
Mono-potassium phosphate	2.2 g

	Glycerol	4 ml
(f)	**Super broth (per liter)**	
	Tryptone	12 g
	Yeast extract	24 g
	Di-potassium phosphate	11.4 g
	Mono-potassium phosphate	1.7 g
	Glycerol	5 ml
(g)	**Defined medium (per liter)**	
	Di-sodium phosphate	6.78 g
	Mono-potassium phosphate	3 g
	Sodium chloride	0.5 g
	Ammonium chloride	1 g
	Magnesium sulphate	2 g
	Glycerol	20 ml
(h)	**Modified super broth (per liter)**	
	Tryptone	12 g
	Yeast extract	24 g
	Di-potassium phosphate	11.4 g
	Mono-potassium phosphate	2.2 g
	Sodium chloride	0.5 g
	Magnesium sulphate	2.4 g
	Ammonium chloride	1 g
	Glycerol	20 ml
(i)	**Feed medium for fed batch fermentation (per liter)**	
	Glycerol	400 ml
	Yeast extract	300 g
	Magnesium sulphate	10 g

(j) Trace metal solution (per liter)

$FeSO_4 \cdot 7H_2O$	2.8 g
$MnCl_2 \cdot 4H_2O$	2 g
$COCl_2 \cdot 7H_2O$	2.8 g
$CaCl_2 \cdot 2H_2O$	1.5 g
$CuCl_2 \cdot 2H_2O$	0.2 g
$ZnSO_4 \cdot 7H_2O$	0.3 g
H_3BO_3,	0.02 g
HCl	1 M

(k) Eagles minimum essential medium (EMEM) (per liter)

EMEM	9.4 g
$NaHCO_3$	2.0 g
Streptomycin	100 mg
Penicillin	100000 U
Gentamycin	40 mg
Milli Q water	900 ml

The medium was filtered through 0.22 µm filter and 10% Fetal bovine serum (FBS) [Sigma, USA] was added at the time of use to make complete EMEM.

(l) Overlay Medium Prepared in EMEM

Carboxymethyl cellulose	1.25%
FBS	2%

3.5 Instrumentation

Shaker-incubator (Kuhner AG, Switzerland), Fermentors of working capacity 5, 10 and 100 liters (New Brunswick, USA), Floor model and table top centrifuge (Thermo, USA), Lab (Pellicon mini) and large Scale (Prostak system, Pellicon Spl.) TFF system (Millipore, USA) for Microfiltration, Ultrafiltration and Diafiltration, Agitator bead mill (Willy A Bachofen, Switzerland), Sonicator (Sonics, USA), Lab and pilot Scale Liquid

Chromatography systems (GE Healthcare, Sweden), Spectrophotometer (Thermo, USA), ELISA Reader (Biotek, USA), Electrophoresis and Gel Documentation system (Bio-Rad, France), Freezers, Biosafety cabinet, Incubator, Bioprocess Scale up Facility and Laboratory Animal Facility of this Establishment was used for this study.

3.6 PROTEIN PURIFICATION COLUMNS, RESINS AND FILTRATION DEVICES

Ni-Sepharose and SP sepharose columns [1 ml, 5 ml and 20 ml (16/10) pre-packed] were used for laboratory scale affinity and ion-exchange chromatographic purification of recombinant proteins respectively. Ni-Sepharose and SP sepharose resin, for affinity and ion-exchange purification of proteins using 5 ml and 10 ml gravity flow column, XK 16/20, XK 26/20, XK 26/40, XK 50/30 were used. Superdex 75 10/300 GL and High load 16/60 gel filtration column were used for gel filtration chromatography. All these columns and resins were from GE Healthcare, Sweden. Quik Scale 70/55 Biochromatography pilot scale column was from Millipore, USA. All the micro (0.45 µ) and ultrafiltration (3, 5, 10 and 30 kDa) membranes (50 cm^2, 0.1 m^2, 0.5 m^2 and 20 ft^2), centrifugal cut-off devices (15 and 50 ml) were purchased from Millipore, USA. Dialysis membranes were from Sigma, USA. PVDF membrane for Western blotting was from Millipore USA. Amicon ultra centrifugal filter devices were from Millipore, USA.

3.7 VIRUS, CELLS, SERUM SAMPLES & EXPERIMENTAL ANIMALS

Dengue virus type 1 to 4 and Japanese encephalitis virus were previously obtained from National Institute of Virology, Pune, India. Cell lines (LLC-MK2, Vero and $C_{6/36}$) were previously obtained from National Centre for Cell Science, Pune, India. The well characterized serum samples previously collected in Gwalior and Delhi in India (for dengue), Gorakhpur in India (for Japanese Encephalitis) and healthy serum samples are included in this study for characterization of proteins. BALB/c mice (CDRI Strain) between 3-4 weeks of age were obtained from the animal facility of DRDE, Gwalior. Viruses, cells, serum samples and CSF samples used in this study were obtained whenever required from Division of Virology of this Establishment.

3.8 REAGENTS AND BUFFERS

All reagents and buffers for protein work were prepared in Milli Q grade water. These are general reagents and buffers which have been used throughout the whole work, although specific things used in particular parts of it can be also found in the corresponding chapters.

Reagents and Buffers for SDS-PAGE

(a) Acrylamide (30%)

Acrylamide	29.2 g
N, N' methylene bisacrylamide	0.8 g
Milli Q water	60 ml

The solution was stirred to dissolve the acrylamide. The volume was made up to 100 ml and the solution was filtered through Whattman filter paper no. 1 before use.

(b) Ammonium per sulfate 10% (w/v)

(c) TEMED 8.4% (v/v)

(d) Resolving buffer

Tris base	9.08 g
SDS	0.2 g

Dissolve in 60 ml of distilled water and adjust pH to 8.8 with concentrated HCl and make up to 100 ml.

(e) Stacking buffer

Tris base	3.03 g
SDS	0.2 g

Dissolve in 60 ml of distilled water and adjust pH to 6.8 with concentrated HCl and make up to 100 ml.

(f) 10X Tris-Glycine buffer (per liter)

Tris base	30.3 g
Glycine	144.1g
SDS	10 g

(g) Coomassie brilliant blue staining solution (per liter)

Brilliant blue (R250)	1 g
Acetic acid	100 ml
Methanol	400 ml
Milli Q water	500 ml

(h) Destaining solution (per liter)

Methanol	400 ml
Glacial acetic acid	100 ml
Milli Q water	500 ml

(i) Lysis Buffer (SDS-PAGE sample buffer)

Tris base	1.51 g
SDS	4 g
Glycine	20 g
Bromophenol blue	0.002 g

Dissolve in 60 ml of water and make up to 90 ml. Add 10 m1 β-mercaptoethanol just before use.

Reagents and buffers for Western blot Analysis

(a) Transfer buffer (per liter)

Tris Base	3 g
Glycine	14.4 g
Methanol	200 ml

(b) Blocking buffer 5% BSA in PBS

(c) Wash buffer 0.05% Tween 20 in PBS (PBS-T)

(d) Developing buffer

DAB	4 mg
0.1 M Citric acid	2.57 ml
0.2 M Na_2HPO_4	2.43 ml
MQ water	5 ml
H_2O_2	15 µl

Reagents for ELISA

(a) Coating buffer (pH 9.6)

Na_2CO_3	0.159 g

	$NaHCO_3$	0.290 g
	Milli Q water	100 ml
(b)	**Blocking buffer**	2% BSA in PBS
(c)	**Wash buffer**	0.05% Tween 20 in PBS (PBS-T)
(d)	**Citrate Phosphate buffer (pH 5.0)**	
	0.1 M Citric acid	2.57 ml
	0.2 M Na_2HPO_4	2.43 ml
	Milli Q water	5 ml
(e)	**Developing buffer for micro plate#/dipstick* ELISA**	
	Ortho Phenylene diamine#/DAB*	4 mg
	0.1 M Citric acid	2.57 ml
	0.2 M Na_2HPO_4	2.43 ml
	MQ water	5 ml
	H_2O_2	15 µl

Buffers for Processing of *E. coli* cell pellet for protein purification

(a)	**Cell wash buffer (pH 8.0)**	
	Tris–HCl	10 mM
	NaCl	100 mM
	EDTA	10 mM
(b)	**Cell lysis buffer (pH 7.5)**	
	Tris HCl	10 mM
	NaCl	100 mM
	EDTA	5 mM
	β-Mercaptoethanol	5 mM
	Benzamidine HCl	5 mM
	PMSF	1 mM
	Lysozyme	100 µg/ml

(c) IB Wash buffer 1 (pH 6.0)

NaH_2PO_4	50 mM
EDTA	5 mM
NaCl	200 mM
Urea	1 M
Triton X-100	1%

(d) IB Wash Buffer 2 (pH 6.0)

NaH_2PO_4	50 mM
EDTA	1 mM
NaCl	1 M

(e) Solubulization buffer (pH 8.0)

Tris HCl	10 mM
NaH_2PO_4	100 mM
β-Mercaptoethanol	20 mM
NaCl	100 mM
Urea	8 M

Buffers for Purification of Recombinant Protein by affinity Chromatography under denatured Conditions

(a) Equilibration buffer (pH 8.0)

Tris HCl	10 mM
NaH_2PO_4	100 mM
NaCl	100 mM
Urea	8 M

(b) Column wash buffer (pH 6.3)

Tris HCl	10 mM
NaH_2PO_4	100 mM
NaCl	100 mM
Urea	8 M

(c) Elution buffer (pH 4.3)

Tris HCl	10 mM
NaH_2PO_4	100 mM
NaCl	100 mM
Urea	8 M

Buffers for On-column refolding using affinity chromatography

(a) Refolding Buffer A (pH 8.5)

Phosphate buffer	50 mM
Urea	3 M
NaCl	300 mM
Glycerol	12% (v/v)

(b) Refolding Buffer B (pH 8.5)

Phosphate buffer	50 mM
Urea	3 M
NaCl	300 mM
Glycerol	12% (v/v)
Imidazole	50 mM

(c) Refolding Buffer C (pH 8.5)

Phosphate buffer	50 mM
Urea	3 M
NaCl	300 mM
Glycerol	12% (v/v)
Imidazole	300 mM

Buffers for Dialysis and Diafiltration

(a) Diafiltration buffer 1 (pH 6.0)

Urea	1 M
Phosphate Buffer	50 mM

(b) Diafiltration buffer 2 (pH 6.0)

Phosphate Buffer	50 mM
NaCl	10 mM

Buffers for salt and pH based Ion-Exchange chromatography

(a) Ion exchange buffer 1 (pH 5.8)-Salt based IEX

Phosphate buffer	50 mM
Sodium chloride	50 mM

(b) Ion exchange buffer 2 (pH 6.0)-Salt based IEX

Phosphate buffer	50 mM
Sodium chloride	500 mM

(c) Ion exchange buffer 3 (pH 6.0)-pH based IEX

Phosphate buffer	50 mM
Sodium chloride	10 mM

(d) Ion exchange buffer 4 (pH 8.0)-pH based IEX

Phosphate buffer	50 mM

3.9 EXPRESSION OF ENVELOPE DOMAIN III PROTEIN OF JE AND DENGUE VIRUSES AND NS1 PROTEIN OF JE VIRUS

E. coli BL21(DE3) cells harboring the pET-30a+ plasmids containing domain III gene for the expression of recombinant JE virus domain III protein; BLR (DE3) cells harboring the pET-30a+ plasmids containing domain III gene for the expression of domain III protein of dengue virus type 1 to 4 and SG13009 cells harboring the pQE30UA plasmid containing NS1 gene for the expression of NS1 protein of JE virus were grown in Luria bertani broth with 50 μg/ml kanamycin [additionally 12.5 μg/ml tetracycline for BLR(DE3) and 100 μg/ml of ampicillin for SG13009] at 200 rpm, 37°C. Briefly, glycerol stock of 1.5 ml was inoculated into 50 ml of LB medium with antibiotics and allowed to grow at 37°C in a incubator-shaker at 180 rpm overnight. About 1% (v/v) of overnight grown culture was inoculated into 100 ml of LB medium in 500 ml Erlenmeyer flasks. At mid-logarithmic phase, the cultures were induced with 1 mM isopropyl *β*-thiogalactopyranoside (IPTG) for

the expression of envelope domain III protein of dengue and JE viruses and NS1 protein of JE virus. Following induction, the cells were grown for 4 hours. The various culture conditions viz. induction time (mid logarithmic phase, stationary phase), inducer concentration (0.5, 1, 1.5, 2 mM), duration of induction (1-4 hours) and inoculums (1-5%) were optimized for maximum expression of proteins. Cultures were harvested by centrifugation at 6000 rpm for 30 min and pellet was recovered. All the samples were lysed in SDS-PAGE sample buffer and analyzed by SDS–PAGE. Un-induced control cultures were analyzed in parallel and the protein concentration was determined by densitometry.

3.10 OPTIMIZATION OF MEDIA FOR PRODUCTION OF ENVELOPE DOMAIN III AND NS1 PROTEINS

Shake flask cultivation was performed in different media in order to find optimal growth conditions. Duplicate cultures were carried out in different media viz. Luria Bertani broth, SOB, SOC, M9 minimal salt, Terrific broth, Super broth (SB), modified super broth and defined medium. A frozen culture (1.5 ml) of recombinant cells was inoculated into 50 ml of primary culture medium (sterile LB medium) containing respective antibiotics in a 250 ml Erlenmeyer flask and incubated for 8 h in an Incubator-shaker (Kuhner AG, Switzerland) at 180 rpm, 37^{o}C. Primary seed culture (1 ml) was used to inoculate 100 ml of secondary culture medium (sterile corresponding medium) in 500 ml Erlenmeyer flasks and grown for 15 h and 37ºC at 180 rpm. For media optimization, secondary seed cultures were used to inoculate at 2% (v/v) in a 1 liter flask containing 200 ml of each media and incubated at 200 rpm and 37ºC in Shaker-Incubator. At mid-logarithmic phase, the cultures were induced with 1 mM isopropyl β-thiogalactopyranoside (IPTG) for the expression of these proteins and further grown for 4 hours before harvesting. The cultures were centrifuged at 6000 rpm for 30 min and the cell pellet was stored for further use. Cultures were analyzed for optical density at a wavelength of 600 nm (OD_{600}), dry cell weight as well as protein yield. The medium showing maximum protein yield among tested media was further used for the production of these proteins by batch and fed batch fermentations. Evaluation of effect of various culture media components viz. glycerol (5-30 ml/l), yeast extract (5-30 g/l) and magnesium sulphate (1.2-2.4 g/l) in combination with above media on cell growth was also carried out as procedure described above.

3.11 BATCH FERMENTATION FOR PRODUCTION OF ENVELOPE DOMAIN III AND NS1 PROTEINS

Laboratory scale batch fermentations were carried out in a bench top fermentor (Bioflo 3000, New Brunswick, USA) equipped with a 5.0 liter working volume glass vessel and stainless steel head plate. Biocommand Plus software was used for data acquisition and operation of the fermentors in batch mode. About 4.0 liters of culture medium was added to the vessel and sterilized by exposure to 121^{o}C, 15 psi for 20 minutes. The dissolved oxygen (DO) probe and pH probe were also inserted and all addition and sampling ports were sealed or clamped and wrapped in aluminum foil before sterilization. A preinoculation sterility check was conducted for a minimum of 15 hours during which pH, agitation and temperature were continuously monitored. After 15 hours, the DO was also monitored along with turbidity. The vessel was adjudged to be sterile if the pH and DO remained constant and no increase in turbidity was observed. The calibration of pH was done before sterilization by using standard buffer pH 7 and 4. Dissolved oxygen probe (Mettler, Toledo, Germany) was calibrated before sterility checking procedure in fermentor as per manufacturer protocol. The initial culture was started in shake flasks by inoculating 1.5 ml of glycerol stock of *E. coli* in 50 ml culture medium with respective antibiotics in 250 ml Erlenmeyer flask. The culture was shaken at 37^{o}C for 8 hours at 180 rpm. About 1% (v/v) culture was added in 200 ml of medium with antibiotics in 1 liter Erlenmeyer flask. This secondary seed culture was incubated at 37^{o}C for overnight at 180 rpm. Once the sterility of the vessel had been verified, the 5% (v/v) overnight grown culture was added to the vessel with respective antibiotics through the addition port of the headplate and the initial OD_{600} was recorded. The initial batch culture conditions in fermentor were as follows: initial culture volume 4.0 liter; temperature 37^{o}C; air flow rate 4.0 liter per minute or 1 vvm (volume/volume/minute); stirrer speed, 200 rpm. Agitation was regulated between 200 and 600 rpm and aeration between 0.5 and 2.0 vvm to keep the dissolved oxygen level in an appropriate level. DO was analyzed using a DO probe and was controlled at about 30% of air saturation by control of both airflow and stirrer speed. If necessary, the inlet air was enriched with pure oxygen using an automated thermal mass flow controller. The temperature was controlled by a heating sleeve and an integrated cooling system to 37°C throughout the process. The pH was controlled between 6.8-7.0 by addition of 25% (v/v) aqueous ammonia or 1N HCl solution. Foaming

was controlled by the addition of antifoam A (Sigma) emulsion. The cell density and dry cell weight analysis were carried out by manual sampling of the fermentor culture through sampling port. The optical density (OD_{600}) of the culture was measured using spectrophotometer at different time interval. Analysis of dry cell weight per 10 ml of culture with respect to OD_{600} revealed that the relationship between the both parameters was linear, confirming that OD_{600} accurately measured cell density. At mid-log phase the fermentor culture was induced with IPTG to a concentration of 1mM. Following induction the culture was further grown for 4 hours before harvesting. Finally the culture was centrifuged at 6000 rpm for 30 min using floor model centrifuge and the cell pellet was stored for further use.

3.12 FED BATCH FERMENTATION FOR PRODUCTION OF ENVELOPE DOMAIN III AND NS1 PROTEINS

The laboratory scale high cell density fed-batch fermentations were performed at 37°C in a fermentor of 10.0 liter working volume (Bioflo 3000, New Brunswick, USA) equipped with Biocommand plus software system for control and data acquisition. The secondary seed culture as grown above (5% v/v) was inoculated to the media in the bioreactor vessel with respective antibiotics through the addition port and the optical density of the preculture was measured to obtain about 0.1-0.2 (OD_{600}) at the onset of the batch phase. The initial batch culture conditions were as follows: initial culture volume, 8.0 liter; temperature, 37^{o}C; air flow, 0.5 vvm; stirrer speed, 200 rpm. Optimized culture media were used as batch culture medium. Mixing of air and oxygen was done using inbuilt thermal mass flow controller. A polarographic electrode was used to analyze the concentration of dissolved oxygen, which was maintained at about ~30% of air saturation. Agitation was varied between 200 and 900 rpm and aeration between 0.5 and 1.5 vvm. During high cell density, the inlet air was enriched with pure oxygen, subject to the oxygen demand of the bacterial culture. The pH was maintained at 6.8-7.0 similar to batch fermentations. All controls were carried out by the digital control unit of the bioreactor. After depletion of the initial nutrients in the batch medium, as indicated by an increase in the dissolved oxygen concentration or increase in pH, feeding of feed medium containing yeast extract and glycerol with magnesium sulphate was then started using inbuilt peristaltic pump in bioreactor console to achieve constant DO (DO stat feeding) and constant pH (pH stat feeding). Foam was detected using the bioreactor foam sensor and suppressed by the addition

of the antifoam reagent similar to batch fermentation. Culture growth was monitored by measuring OD_{600} during process. The culture was induced with 1 mM IPTG at high cell densities and the induction phase lasted for 4 hours. Finally the culture was harvested by centrifugation and the cell pellet was stored for further purification process.

3.13 PILOT SCALE FERMENTATION FOR PRODUCTION OF ENVELOPE DOMAIN III PROTEINS

Pilot scale fermentations were performed on a 100 liter fermentor (Bioflo 5000-New Brunswick, USA) containing 80-85 liter of modified SB medium. A recombinant strain of *E. coli* containing EDIII gene of JE virus or dengue virus type 3 was used as seed culture. About four liter of inoculum cultured in modified SB medium for 6 h either in lab scale fermentor as described above or four Erlenmeyer shake flasks of 5 liter capacity with 1 liter modified SB medium was used as inoculum for a 100 liter fermentor. For inoculum preparation, the primary and secondary culture was grown as above with respective antibiotics. About 2% (v/v) of overnight grown shake flask culture (secondary culture) in modified SB medium was added in each of flasks containing 1 liter of modified SB medium and incubated at 37^{o}C for 6 h at 200 rpm. This seed culture (5% v/v) or 4 liter of 6 h grown batch fermentation culture was added aseptically to the media in the 100 liter fermentor vessel with respective antibiotics. It is important to monitor and control the following parameters throughout the fermentation process: temperature; DO; pH; agitation; aeration; foaming. The pilot scale fermentations were performed with following set-up: initial volume, 80-85 liter; air flow rate, 0.25-0.9 vvm; stirrer speed, 50-350 rpm; pH, 6.8-7.0; temperature, 37^{o}C and dissolved oxygen, ~30%. The DO level was maintained at about 30% saturation using air, pure oxygen and stirring speed of up to 350 rpm. The pH of the medium was maintained by automatic addition of ammonia and phosphoric acid. Anti-foam was added as required. At mid log phase (after ~five and half hour of culture growth), IPTG was added as inducer into the fermentor vessel at a concentration of 1 mM and the total duration of induction was 4 hours. Sampling of the culture medium was performed during fermentation for measurement of OD_{600}. The fermentation broth was harvested using Prostak TFF microfiltration system (Millipore) with 0.45 µm pore size of 20 square foot area membrane. After microfiltration, the concentrated broth was further centrifuged at 6000 rpm for 30 min using floor model centrifuge. After centrifugation, the cell pellet was stored for further use.

3.14 CELL DISRUPTION AND SOLUBILISATION OF INCLUSION BODIES

The cell pellet was washed twice with cell wash buffer (1:20, w/v) by centrifugation at 10,000 rpm for 20 min at 4^{o}C. The washed pellet was re-suspended in chilled cell lysis buffer (1:20, w/v). The cell suspension was disrupted by passing it three times through agitator bead mill at the flow rate of 50 ml/ min in 0.6 liter container at 1 bar pressure or by sonication using a sonicator (Sonics, USA) at a setting of 70% frequency using high gain probe. The sonicator was programmed to provide 9 s pulses with 9 s pause for a total period of 10 min. Lysate was centrifuged at 10,000 rpm for 40 min at 4°C and supernatant removed. The cell pellet containing the inclusion bodies (IBs) was washed with IB wash buffer 1 (1:20, w/v) followed by another wash with IB wash buffer 2 by centrifugation at 10,000 rpm for 40 min at 4^{o}C. The supernatant was carefully removed and the IB pellet was stored until further use. The IB washed pellet containing inclusion bodies was used for further purifications.

Inclusion bodies were solubilized in solubilization buffer (1:20 v/w) using a mechanical homogenizer (Kinematica AG, Switzerland) at 5000 rpm for about 20 min or stirred on a magnetic stirrer for 6-8 hours at room temperature. This suspension was then centrifuged at 10,000 rpm for 40 min at 4°C as well as filtered with 0.45 µm membrane (Millipore, USA). The supernatant containing the solubilized IBs was collected and its protein concentration was determined by the BCA assay. We also estimated the total protein by taking absorbance at 280 nm in spectrophotometer and calculating the protein concentration using absorbance coefficient of EDIII proteins. This clear supernatant was used for affinity chromatography under denaturing conditions or on-column refolding of these proteins.

3.15 PURIFICATION OF ENVELOPE DOMAIN III AND NS1 PROTEIN USING AFFINITY CHROMATOGRAPHY

For small scale immobilized metal affinity chromatographic purifications, pre-packed Ni-sepharose columns were used. AKTA pilot and AKTA explorer chromatography systems were used for the large scale and lab scale purification of proteins. For large scale purifications streamline chelating resin (GE Healthcare) was packed in an XK16/20 (20 ml

resin) or quik scale 70/55(500 ml resin) column. For packing the streamline chelating resin was poured into the column in one continuous motion. The inlet and outlet connections of column were connected to a chromatography system. The column was charged with three column volumes of 50 mM $NiCl_2$ solution. The column was continuously washed with five column volumes of distilled water. The filtered solution containing denatured protein was loaded to the chromatography column using AKTA chromatography system. The clear supernatant was loaded at a linear flow rate of 150 cm/h to the chromatography column. The packed column was pre-equilibrated with solubilization buffer before applying clear supernatant. After loading, the column was washed with column wash buffer at pH 6.3 and 6.0, at a linear flow rate of 150 cm/h. The bound protein was eluted using elution buffer at pH 4.3. The elution buffer has similar composition as equilibration buffer, but the pH was different. The relevant elutes were pooled together and dialyzed/diafiltered successively against dialysis/diafiltration buffer (50 mM phosphate buffer) containing progressively decreasing urea concentration (6 M, 4 M, 2 M, 1 M and 0 M). This purified protein was used as an antigen in ELISA for detection of JE/Dengue infections.

3.16 REFOLDING WITH SIMULTANEOUS PURIFICATION OF ENVELOPE DOMAIN III PROTEIN

For vaccine studies of envelope domain III protein, refolding is necessary to achieve native conformations. Immobilized metal affinity chromatography using AKTA pilot system (GE Healthcare, Sweden) was used for the large scale refolding with simultaneous purification of rJEV EDIII and rDen 3 EDIII proteins. For small scale, Akta explorer system was used with 5ml or 16/10 prepacked IMAC column. Large scale column were packed and charged as described above. Three buffers were used in the simultaneous refolding and affinity chromatographic process: on-column refolding buffer A, buffer B and buffer C. The clarified supernatant containing EDIII protein was directly loaded onto a column containing Ni charged resin equilibrated previously with the buffer A. The flow rate was 150 cm/ h. After loading, unbound proteins were removed by washing the column with five column volume of the buffer B. The EDIII protein was eluted with Buffer C.

3.17 DIAFILTRATION, SALT AND PH BASED ION-EXCHANGE CHROMATOGRAPHY

The eluted fractions were analyzed by SDS–PAGE and fractions having majority of EDIII protein band were pooled, concentrated using ultrafiltration device (Pellicon-Millipore) with a 3-kDa cut-off ultrafiltration cassette (Millipore) and buffer exchanged against solution containing 50 mM phosphate buffer, 1 M urea, pH 6.0. The SP sepharose cation exchange resin packed in an XK 26/40 (150 ml) column or prepacked SP sepharose column (5 ml and 16/10) was used for cation exchange chromatographic purifications of proteins. After packing, the column was washed with five column volumes of distilled water and then equilibrated with ion exchange buffer 1.

For salt based IEX, refolded and diafiltered sample was loaded onto the column containing pre-equilibrated SP- Sepharose cation exchange resin. Column was washed with ion exchange buffer 1 and bound proteins were eluted with ion exchange buffer 2. The elutes were pooled, buffer exchanged with 50 mM phosphate buffer pH 6.0, 10 mM NaCl by diafiltration using a 3-kDa cut-off, ultrafiltration device and processed further for ion exchange chromatography using pH. This diafiltered protein sample was loaded onto the SP sepharose column matrix pre-equilibrated with ion exchange buffer 3 and EDIII protein was eluted from the column using ion exchange buffer 4. The fractions were analyzed on SDS-PAGE gel and relevant fractions containing EDIII protein were pooled, filter sterilized, and stored at -80^{o}C until further use.

3.18 GEL FILTRATION CHROMATOGRAPHY

Gel filtration (GF) chromatography was used after IMAC or IEX chromatography for further purification for envelope domain III proteins if required. Superdex 75 (10/300 GL) or 16/60 Hi-load gel filtration column was used for this purpose. The column was equilibrated with 50 mM phosphate buffer pH 5.8 or pH 8.0 at a constant linear flow rate of 50 cm/h. Purified protein was loaded to the pre-equilibrated Superdex 75 column at a linear flow rate of 50 cm/h. Elution of purified protein from the column was done using same buffer and monitored at 280 nm. Elutes were analyzed by SDS-PAGE and stored at -80^{o}C until further use.

3.19 OFFLINE MEASUREMENT AND PROTEIN ANALYSIS

3.19.1 Optical Density and Dry Cell Weight

The Cell growth was monitored by measuring optical density, wet cell weight and dry cell weight. Optical density of the culture was measured at 600 nm (OD_{600}) with UV-visible spectrophotometer. Samples with higher values of OD_{600} were diluted suitably to have OD in the range of 0.2–0.4. In order to obtain the dry cell weight, 10 ml of culture broth was centrifuged at 8,000 rpm for half an hour and washed with distilled water in pre weighed tubes. After draining the supernatant, the tube with the cell pellet was weighed on a balance to determine the wet cell weight. To determine the dry cell weight, the same tube was left overnight in an incubator at 105^{o}C and was weighed next day till constant weight.

3.19.2 Sodium Dodecyl Sulphate–polyacrylamide Gel Electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) using a Mini-protean III apparatus (Bio-Rad) were used to monitor the purity of protein after chromatographic procedures. Electrophoresis was performed at 60 V using 12% polyacrylamide gels. Detection was performed with Coomassie brilliant blue and silver staining.

3.19.3 Determination of Protein Concentration by Bicinchoninic Acid (BCA) Assay

The BCA protein assay is a detergent-compatible formulation based on BCA for the colorimetric detection and quantification of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing BCA. The purple coloured reaction product of this assay is formed by chelation of two molecules of BCA with one cuprous ion. This water soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000 µg/ ml). Working reagent was prepared by mixing 49 parts of BCA reagent A and 1 part of BCA reagent B. About 25 µl of each standard or unknown sample replicates were pipetted into a micro plate well. About 200 µl of working reagent added to each well and mixed thoroughly. The plate was covered and incubated at 37°C for 30 min and absorbance was measured at 560 nm on plate reader. A standard curve was plotted by taking known concentrations of Bovine serum albumin (BSA)

ranging from 20-2000 µg/ml and the concentration of test protein sample was calculated from the standard curve.

3.19.4 Analysis of Protein Purity by Silver Staining

For determination of protein purity 500 ng of purified protein was resolved on 12% SDS-PAGE and subjected to silver staining using Silver staining kit (Fermentas, USA) as per manufacturer's instructions. Briefly after gel electrophoresis, the gel was placed in a fixing solution with gentle agitation for 20 min. Fixing solutions were decanted and gel was rinsed in distilled water. Then gel was placed in sensitizing solution with gentle agitation. After gel washing with distilled water, staining solution was added and gel was stained for approximately 20 min or until desired staining intensity was reached. After staining developing solution was added to develop the protein band and reaction was stopped by addition of stopping solution for a minimum of 15 min. After stopping the reaction, the gels were rinsed in distilled water for 5 min.

3.19.5 Western Blotting

For Western blot analysis, the purified protein was run on 12% denaturing gel (SDS–PAGE), along with pre-stained protein markers (Fermentas, USA) on adjacent lanes and transferred electrophoretically to polyvinylidene difluoride (PVDF) membrane. The unoccupied sites on PVDF membrane was blocked with 5% BSA (Bovine Albumin Serum) in PBS (Phosphate Buffer Saline) and incubated at 4^{o}C overnight. Blocked membranes were washed three times each with duration of 5 min with PBS containing 0.05 % Tween-20 (v/v) and incubated at 37^{o}C with IgG antibodies positive serum sample (1:100 dilution in PBS, v/v) or anti-histidine antibodies or hyper immune serum (1:5000 dilution in PBS, v/v) raised in mice against EDIII proteins for 1 hour. The membrane was washed again as above and incubated with Goat anti mouse or anti human IgG horseradish peroxidase (HRP) conjugate for 1 h at 37^{o}C in 1:1000 in PBS (v/v). The membrane was washed again and developed by incubation in developing buffer containing DAB (Diaminobenzidine)/H_2O_2 substrate for 15 min at 37^{o}C.

3.20 EVALUATION OF DIAGNOSTIC POTENTIAL OF ENVELOPE DOMAIN III AND NS1 PROTEIN

The affinity purified and dialyzed/diafiltered domain III/NS1 protein was used in ELISA for evaluation of its diagnostic potential. The purified protein was evaluated for the

detection of IgM and IgG antibodies from dengue suspected patient's serum samples and IgM antibodies form JE suspected serum and CSF samples. A panel of clinical samples comprising serum and cerebrospinal fluid (CSF) samples for JE and serum samples for dengue was included in this study. In addition, a panel of serum samples obtained from 10 healthy persons was also included in this study. In-house indirect microwell plate ELISA (quantitative) as well as indirect dipstick ELISA (qualitative) was carried out for detection of anti-JEV IgM antibodies in serum and CSF samples and anti-dengue IgG and IgM antibodies in serum samples using IMAC purified protein.

3.20.1 Indirect Microwell Plate ELISA

For indirect microwell plate ELISA, purified protein was diluted to attain 0.4 µg (EDIII protein) or 0.5 µg (NS1 protein) per 100 µl in coating buffer (Na_2CO_3 0.1 M, $NaHCO_3$ 0.2 M; pH-9.6) and used for coating 96-well (100 µl/well) micro titer plates (Nunc, Germany) at 37°C for 1 h. The coated wells were washed once with PBS and blocked with 2% BSA in PBS overnight at 4°C. The wells were washed once again with PBS-T washing buffer and then incubated for 1 h at 37°C with 100 µl serum (1:100) and CSF (1:10) samples in PBS +2% BSA with 0.01% Tween-20 separately. Wells were washed three times using wash buffer and incubated with anti-human IgM HRP conjugate (1:2,000 dilution in PBS+2% BSA). The wells were washed once again as above and incubated with 100 µl of citrate-phosphate buffer with OPD substrate for 10 min at 37°C. Peroxidase reaction was terminated with 100 µl of 1N H_2SO_4 and the absorbance was read at 490 nm using ELISA reader (Biotek Instruments, USA). The in-house microplate ELISA for anti-dengue IgG antibodies was performed as above with patient serum samples (1:1000 dilution) and Goat anti-human IgG horseradish peroxidase (HRP) conjugate (1:3000 dilution). Suitable positive and negative controls were always included while performing the test.

3.20.2 Indirect Dipstick ELISA

The in-house indirect dipstick ELISA for detection of anti-JE IgM and anti-dengue IgM and IgG antibodies was also carried out. For this purpose, the purified protein was coated onto nitrocellulose (NC) combs (MDI, India) with 12 projections, at the rate of 0.4 µg (EDIII protein) or 0.5 µg (NS1 protein) per 2 µl coating buffer per projection and incubated at 37^{o}C for 1 h. The unoccupied sites were blocked with 2% (BSA) in PBS and incubated at 4^{o}C overnight. These coated NC projections were washed with PBS-T wash buffer. Serum

(1:100) and CSF (1:10) samples were diluted with serum diluents (0.01% Tween-20 in PBS+2% BSA) and dispensed at 200 µl/well. The antigen coated combs were dipped in the wells of this microtiter plate and incubated for 1 h at 37^{o}C. Combs were then washed with wash buffer three times, each with duration of five min. After washing, the combs were dipped in Goat anti-human IgM HRP conjugate, diluted (1:2000) in conjugate diluent (PBS with 2% BSA), and incubated for 1 h at 37^{0}C. The combs were then washed as above and developed with phosphate-citrate buffer containing diaminobenzidine (DAB) and H_2O_2. Appearance of brown colour dot indicated presence of IgM antibodies. The in-house dipstick ELISA for anti-dengue IgG antibodies was performed as above with patient serum samples (1:1000 dilution) and Goat anti-human IgG horseradish peroxidase (HRP) conjugate (1:3000 dilution). Suitable positive and negative controls were always included while performing the test.

All the serum and CSF samples for JE were also tested by JEV Chex IgM capture ELISA (XCyton, India) and Pan-Bio IgM capture ELISA (Pan-Bio, Australia) as per manufacturer's protocol. All the serum samples for dengue were tested by using Pan-Bio IgM and IgG capture ELISA and Pan-Bio IC test as per manufacturer's protocol. The results of in-house ELISA were compared with findings of commercial test. The in-house ELISA tests were also carried out with healthy serum samples to check non-specific reactivity.

3.21 BIOLOGICAL ACTIVITY OF ENVELOPE DOMAIN III PROTEIN FOR POSSIBLE VACCINE POTENTIAL

3.21.1 Biological Activity of EDIII Protein by ELISA

The biological activity of the EDIII protein was determined by ELISA. The pH based IEX purified EDIII protein was used for raising hyper immune sera in a group of six BALB/c mice. For this purpose, 25 µg of purified protein was injected per mice per dose. The techniques used for bleeding, and sacrifice of animals were strictly performed following mandates approved by the animal ethics committee (Committee for the Purpose of Control and Supervision of Experiments on Animals, Govt. of India).The endotoxin content of the purified and refolded EDIII protein was determined by Limulus Amebocyte Lysate (LAL) QCL-1000 kit (Cambrex, USA). The endotoxin content of purified protein was less than 5EU per 25 µg of EDIII antigen, the dose used per immunization, as determined using standard

LAL assay. The EDIII protein was emulsified with FCA (Freund's Complete Adjuvant) in 1:1 ratio. For subsequent booster doses, EDIII was formulated with FIA (Freund's Incomplete Adjuvant) instead of FCA. The control group of mice was injected with adjuvant only in phosphate buffer saline (PBS). About 200 µl of emulsified EDIII protein (25 µg) was injected subcutaneously at interval of 0, 21 and 42 days. After 14^{th} day of last inoculation the mice were bled and serum was collected and stored at $-20^{o}C$ for use in ELISA and Western blotting.

Sera collected from mice were tested for the recognition of EDIII protein by ELISA. Microtitre ELISA plate (Nunc, Germany) was coated with this protein (400 ng per 100 µl per well) in coating buffer and incubated at $37^{o}C$ for 1h. The plate was washed with phosphate buffered saline containing 0.05% Tween 20 (PBST) and blocked with 200 µl of 5% bovine serum albumin in PBS for overnight at $4^{o}C$. Test sera serially diluted two fold in PBS starting with 1:1000 were incubated in triplicate wells (100 µl/well) at $37^{o}C$ for 1h. The wells were washed three times with PBST wash buffer. Goat Anti-mouse (IgG) antibodies conjugated to horseradish peroxidase were diluted 1:2500 in 2% BSA with PBS, added to wells (100 µl per well) and incubated for 1h at $37^{o}C$. The reaction was developed at room temperature with 100 µl of developing buffer containing orthophenylenediamine dihydrochloride (Sigma) as chromogen and hydrogen peroxide as the substrate. The reaction was stopped with 2 N sulfuric acid. The absorbance was measured at 490 nm using an ELISA plate reader. Mean A490 values for each group were calculated. Sera from individual mice in each group are used for the determination of ELISA end point titers. Dilutions of sera at which A490 is 2 times the background (A490 using sera from day-1, blank wells, or sera from adjuvant controls) were considered to be the ELISA end point titers.

3.21.2 Plaque Reduction Neutralization Test (PRNT)

Sera collected from mice immunized with envelope domain III protein in combination with FCA or pre-immune sera (control) were evaluated using plaque reduction neutralization test to determine the neutralizing ability of these antibodies to JE virus or dengue virus type 3 infectivity. Two hundred microlitres of serum in two fold serial dilutions (from 1:2 to 1:64) were prepared in eppendorf tubes. JE or dengue virus type 3 stock adjusted to 50 pfu in virus diluent [10% Hank's balanced salt solution (HBSS), 0.1% BSA; pH 7.2-7.4] was added to the tube containing serially diluted antibodies. The antibody and virus

dilutions prepared were mixed, pulse centrifuged and then incubated at 37°C for 1 h. A 24 well plate with confluent monolayer of Vero cells for JE virus and LLC-MK2 cells for dengue virus was used for virus infection. Before inoculation of the antibody-virus mixture, the cell monolayer was rinsed once with PBS, after which 200 µl of the antibody-virus mixture was added to the appropriate wells. The plates were left at 37°C for 1 h, and rocked at 15 min intervals. After incubation, the inoculm was removed and the cell monolayer was rinsed once with virus diluent. Overlay medium (1.25% carboxymethyl cellulose in EMEM containing 2% FBS) was added and incubated further at 37°C for 6 days, and virus plaques were stained with 0.2% crystal violet.

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CHAPTER 4

PRODUCTION OF RECOMBINANT JEV EDIII PROTEIN BY BATCH AND FED-BATCH FERMENTATION AS WELL AS EVALUATION OF ITS DIAGNOSTIC POTENTIAL

ABSTRACT

Japanese encephalitis (JE) is one of the leading causes of acute encephalopathy affecting children and adolescents in the tropics. Optimization of media was carried out for enhanced production of recombinant JE virus envelope domain III (EDIII) protein in *Escherichia coli*. Further, batch and fed-batch cultivation process in *Escherichia coli* was also developed in optimized medium. Expression of this protein in *E. coli* was induced with 1 mM IPTG and yielded an insoluble protein aggregating to form inclusion bodies. The inclusion bodies were solubilized in 8 M Urea and the protein was purified under denaturing conditions using immobilized metal affinity chromatography. After fed-batch cultivation, the recombinant *E. coli* resulted in dry cell weight and purified protein about 36.45 gl^{-1} and 910 mgl^{-1} of culture respectively. The purity of the recombinant JE virus EDIII protein was checked by SDS-PAGE analysis and reactivity of this protein was determined by Western blotting and ELISA with JE virus infected human serum samples. These results establish the application of this protein to be used for the diagnosis of JE virus infection or for further studies in vaccine development. This process may also be suitable for the high yield production of other recombinant viral proteins.

4.1 INTRODUCTION

Japanese encephalitis (JE), a mosquito borne viral disease is a major public health problem in South-East Asia and Western Pacific countries. Around 30,000–50,000 cases of JE and up to 15,000 deaths are reported annually (Ghosh and Basu, 2009). Approximately 2.8 billion people inhabit this vast geographical area, where JE is likely to remain an important public health problem in the 21st century (Shukla et al., 2009). Early diagnosis plays a crucial role to forecast an early warning of epidemic and to undertake effective vector control measures. Laboratory diagnosis of JE virus relies on virus isolation, detection of genomic RNA and virus-specific antibodies by RT-PCR and serodiagnosis, respectively (Solomon et al., 1998; Parida et al., 2006). Both virus isolation and RT-PCR are time consuming and costly laboratory methods. Thus, in a majority of cases the only feasible diagnosis is based on the detection of anti-JE IgM antibodies using capture ELISA and indirect ELISA format (Solomon et al., 1998; Ravi et al., 2006; Jacobson et al., 2007; Shrivastva et al., 2008; Ravi et al., 2009; Litzba et al., 2010). However, all these kits are

expensive due to the high costs associated with antigen production, making them unaffordable for use in the economically weaker countries where JE occur most frequently.

Envelope protein of Japanese encephalitis virus constitutes the major immunogen and the major target of the host antiviral immune response **(**Tiroumourougane, 2002). Most of the recombinant DNA-based strategies focus on the envelope (E) proteins of the JE virus. Envelope (E) protein of JE virus is a major antigen responsible for eliciting neutralizing antibody response and protective immunity in hosts (Kaur and Vrati, 2003; Wang et al., 2009; Witthajitsomboon et al., 2010). The E protein is organized into distinct domains designated as I, II and III. Of these, domain III is particularly important from the viewpoint of diagnostic and vaccine development as it contains neutralizing epitopes and the host cell receptor recognition sites (Wu, 2003; Alka et al., 2007; Shukla et al., 2009; Verma et al., 2009)**.**

Escherichia coli is the most commonly used host for heterologous protein production (Lee, 1996; Babu et al., 2000; Khalilzadeh et al., 2004) because its genetics are well understood. Using recombinant *E. coli* in batch and fed-batch cultivations, recombinant proteins have been successfully expressed in *E. coli*. Recombinant *E. coli* can be grown to high densities in common media such as Luria Bertani broth (LB), SOB medium, SOC medium, M9 minimal salt medium and Super broth (SB) medium (Lim et al., 2000; Madurawe et al., 2000; Manderson et al., 2006). To ensure that oxygen supply does not become limiting, the fed-batch operation is used extensively in cultures. This improves biomass and recombinant protein yield relative to batch culture. There is currently a need for developing cultivation process for enhanced production of recombinant Japanese encephalitis virus envelope domain III (rJEV EDIII) protein and development of cost-effective, safe and simple diagnostics with sensitivity, specificity and applicability in laboratory as well as field conditions. In this chapter, we describe the media optimization, high yield production of rJEV EDIII protein in *E. coli* using bioreactor of 10.0 liter working volume, its purification and characterization. Further, we present data demonstrating its utility as a diagnostic reagent by an in-house ELISA in detection of anti-JE IgM antibodies in patient cerebrospinal fluid (CSF) and serum samples.

4.2 MATERIALS AND METHODS

The details about materials used for production of rJEV EDIII protein are desribed in the material and methods section (Chapter 3). The methods for expression, media optimization, small scale batch fermentation, fed-batch fermentation, affinity chromatography, SDS-PAGE, Western blot and ELISA are described in the material and methods section (Chapter 3). A typical flow diagram for production and purification of rJEV EDIII protein used as a diagnostic reagent are shown in figure 4.1.

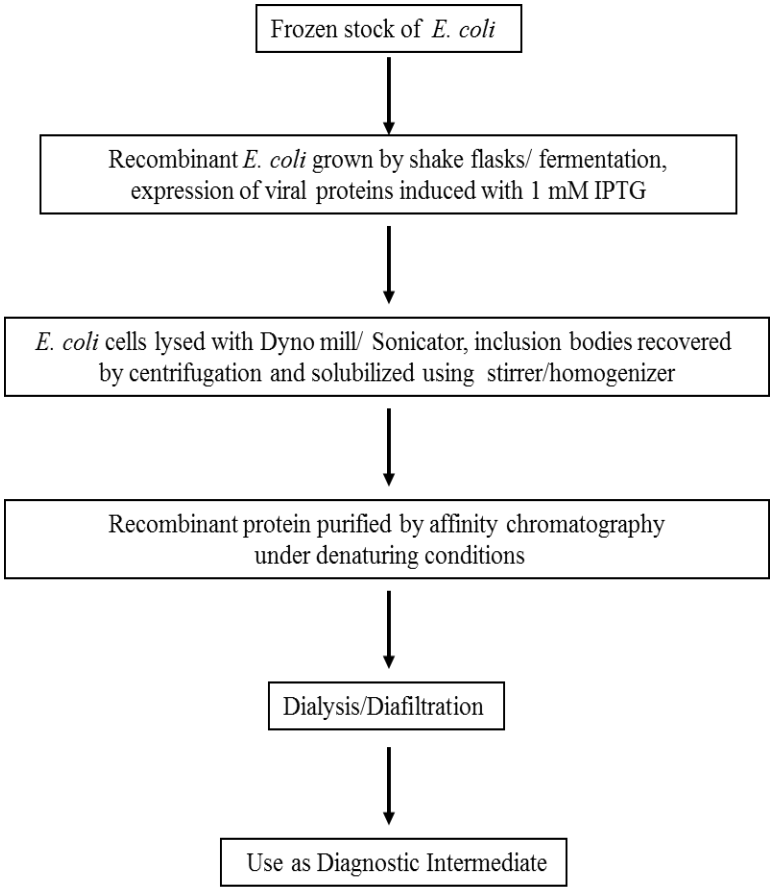


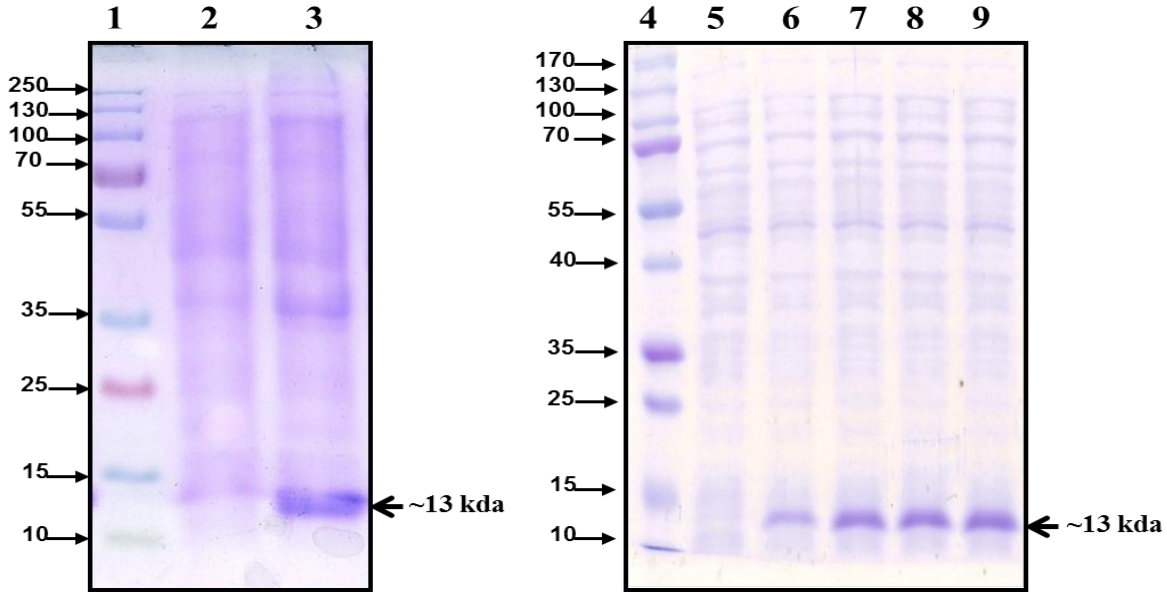
Figure 4.1. Flow diagram of production and purification of rJEV EDIII protein for diagnostic use.

4.3 RESULTS

4.3.1 Expression of Recombinant JE Virus EDIII Protein

To express recombinant JE virus envelope domain III protein in *E. coli*, shake flask culture was carried out in LB medium. A typical induction experiment comparing the profiles

of un-induced and IPTG-induced *E. coli* cultures is shown in figure 4.2.a. It is evident that IPTG induction results in the expression of a unique 13 kDa JEV EDIII protein. IPTG (1 mM) at 4 h post induction time has shown the maximum protein expression (Figure 4.2.b). Therefore, in all subsequent experiments induction was carried out with 1 mM IPTG for 4 h.



(a) (b)

Figure 4.2. SDS-PAGE analysis of expression of rJEV EDIII protein. Lane 1, 4 : Molecular weight marker (kDa) ; lane 2: Un-induced culture ; lane 3 : Induced culture ; lane 5-9: Induced culture of 0, 1, 2, 3 and 4 h respectively.

4.3.2 Effect of Media on Production of rJEV EDIII Protein

In the shake flask culture, the final cell density and cell concentration was found to depend upon the media used. Super broth medium achieved final dry cell weight about 2.55 gl^{-1}. Five times LB (5x) medium produced the final cell density (OD_{600}) of about 0.306. SOB and SOC medium resulted final dry cell weight about 1.43 gl^{-1} and 1.89 gl^{-1} respectively. However, LB medium produced 1.30 gl^{-1} dry cell weight. Growth of this culture in M9 minimal defined medium also resulted OD_{600} of about 0.167. The higher concentration of LB (5x LB) and M9 minimal salt medium provide resistance to the cell growth and resulted in lower cell densities. Comparative yields of purified rJEV EDIII protein in different media are summarized in table 4.1.

Table 4.1. Comparative yield of purified rJEV EDIII protein in shake flask culture.

Media	Dry cell weight (g/l)	Protein (mg/l)
Luria Bertani Broth	1.30	25.17
SOB	1.43	27.23
SOC	1.89	28.86
Terrific Broth	2.40	35.08
Super Broth	2.55	35.55

4.3.3 Production of rJEV EDIII protein in *E. coli*

For maximizing the volumetric production, the *E. coli* cells expressing rJEV EDIII protein were grown in a fed-batch cultivation process. The culture at cell OD of 44.10 was induced with 1 mM IPTG and grown for another 4 h. Production of the maximum amount of rJEV EDIII protein was attained after 4 h post induction. The final dry cell weight of about

Table 4.2. Comparative yield of purified rJEV EDIII protein in bioreactor.

Media	Culture Condition	Dry cell weight (g/l)	Protein (mg/l)
Terrific Broth	Batch fermentation	4.05	112.37
Super Broth	Batch Fermentation	4.16	114.06
Super Broth	Fed-batch Fermentation	36.45	910.23

36.45 gl^{-1} (Table 4.2.) was attained at ~14 h of cultivation in fed batch mode. In batch cultivation with super broth medium, the DCW at induction and harvest was 2.18 gl^{-1} and 4.16 gl^{-1} respectively. The final DCW at harvest using terrific broth medium in batch cultivation was 4.05 gl^{-1}. Real time profile of fed-batch cultivation for production of rJEV EDIII protein is shown in figure 4.3. Comparative yields of rJEV EDIII protein by batch and fed-batch cultivation process is shown in table 4.2.

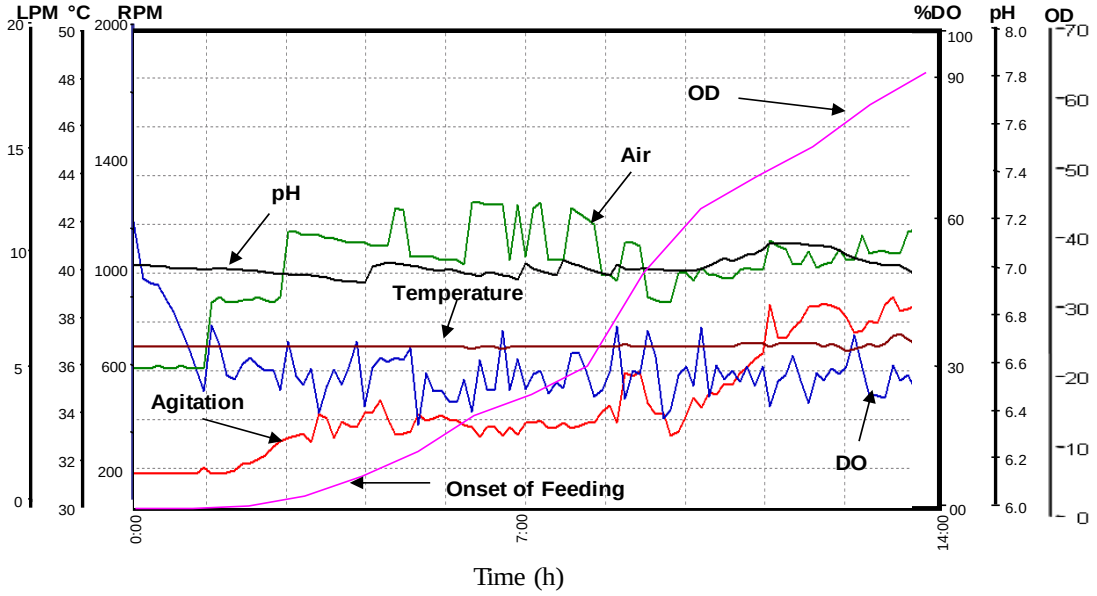


Figure 4.3. Real time profile of fed-batch fermentation for production of recombinant Japanese encephalitis virus envelope domain III (rJEV EDIII) protein. Feeding was started when OD value reaches about 4.91 after ~4.0 h of inoculation.

4.3.4 Purification and Characterization of rJEV EDIII Protein

We have demonstrated the single step purification of rJEV EDIII protein using immobilized metal affinity chromatography. The chromatogram of affinity purification is shown in figure 4.4. The gel analysis showed that more than 95% purity has been achieved (Figure 4.5.a). The purity of this protein was evaluated densitometrically from SDS-PAGE gels using Quantity One image quantification software (Bio-Rad, USA). The final yield of rJEV EDIII protein was ~910 mgl^{-1} (Table 4.2.), which was increased after fed-batch cultivation to about more than thirty six times in comparison with commonly used shake flask culture with LB medium (Table 4.1.).

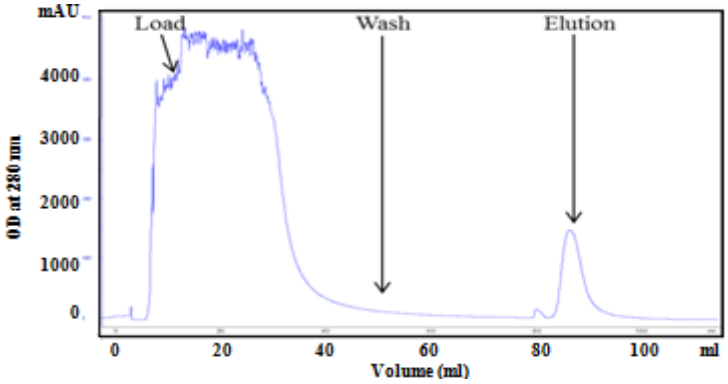


Figure 4.4. Real time profile rJEV EDIII protein purification by affinity chromatography.

Purified rJEV EDIII protein migrates with an appropriate molecular weight of ~13 kDa on denaturing SDS-PAGE gel (Figure 4.5.a). This protein was subjected to Western blot assay, to confirm its identity as rJEV EDIII protein. The affinity purified rJEV EDIII protein was probed with JEV positive hyper immune sera. These results revealed that the JEV positive serum sample reacted with rJEV EDIII protein (Figure 4.5.b). It is evident that the rJEV EDIII protein specifically reacted with anti-JE virus antibody and thus suggests that this protein could be used for the purpose of diagnosis of JE virus infection.

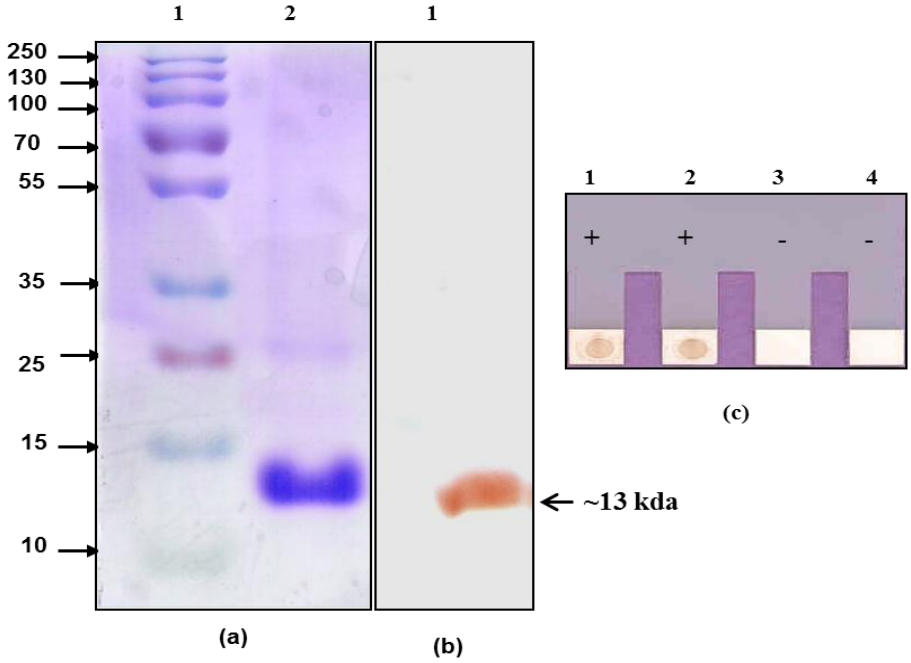


Figure 4.5. (a) SDS–PAGE analysis of final purified rJEV EDIII protein after affinity purification. Lane 1, Molecular Weight Marker (kDa); lane 2, AC purified rJEV EDIII protein. (b) Western blot analysis of the purified rJEV EDIII protein with hyper immune serum sample. Upon 12% SDS-PAGE, the protein was electroblotted and probed with hyper immune serum raised in mice at 1:500 dilution followed by incubation in secondary antibody HRP conjugate (1:1000) and finally developed in DAB/H_2O_2 substrate solution. (c) Results of Dipstick ELISA with rJEV EDIII protein. NC comb coated with 0.4 µg of purified protein was tested with both CSF (1:10 dilution) and serum sample (1:100 dilution) followed by incubation in secondary antibody HRP conjugate (1:2000 dilution) and finally developed in substrate solution (DAB/H_2O_2). Lane 1, IgM positive CSF; lane 2, IgM positive serum; lane 3, negative CSF and lane 4, negative serum samples.

4.3.5 Evaluation of rJEV EDIII Protein by in-house ELISA

In the present study, a total of 80 clinical specimens (50 serum and 30 CSF samples)

was included. All the samples were tested with Pan-bio IgM capture ELISA, JEV Chex IgM capture ELISA and in-house dipstick as well as microwell plate ELISA. Optical density (OD_{450}) values of serum and CSF samples determined with Pan-bio IgM capture ELISA are shown in table 4.3. The cut-off value (OD_{490}) for detection of antibodies using in-house microwell plate ELISA was 0.20 for detection of anti-JEV IgM antibodies in CSF and 0.60 for serum samples. The cut-off value for Pan-Bio capture ELISA was determined by taking average of OD_{450} values of cut-off calibrator in triplicate as per manufacturer's protocol.

Table 4.3. OD_{450} values in Pan-Bio IgM capture ELISA for CSF and serum samples.

Range of OD values[a]	No. of samples	
	CSF	Serum
0.000–0.199	09	20
0.200–0.399	04	08
0.400–0.599	08	10
0.600–0.799	06	07
≥ 0.80	03	05
Total	**30**	**50**

[a]ELISA OD_{450} values of ≥ 0.20 were considered positive as per manufacturer's instruction.

Optical density (OD_{450}) values of serum and CSF samples determined with JEV Chex IgM capture ELISA is shown in table 4.4.

Table 4.4. OD_{450} values in JEV-CheX IgM capture ELISA for CSF and serum samples.

Range of OD values[a]	No. of samples	
	CSF	Serum
0.000–0.699	09	19
0.700-0.799	02	03
0.800–1.19	06	05
1.20–1.59	06	10
1.60–1.99	05	08
≥ 2.00	02	05
Total	**30**	**50**

[a]ELISA OD_{450} values of ≥ 0.70 and ≥ 0.80 were considered positive for CSF and Serum sample respectively as per manufacturer's instruction.

Out of 50 serum samples, 29 samples were positive and 21 samples were negative and of 30 CSF samples 20 were positive and 10 samples were negative by in-house dipstick as well as microwell plate ELISA. Out of 50 serum samples, 30 samples were positive and 20 samples were negative and of the 30 CSF samples 21 were positive and 9 samples were negative by Pan-Bio IgM capture ELISA. Out of 50 serum samples, 31 samples were positive and 19 samples were negative and of the 30 CSF samples 21 were positive and 9 samples were negative by JEV-CheX IgM capture ELISA.

Table 4.5. Comparative evaluation of in-house ELISA with reference to Pan-Bio IgM capture ELISA for detection of anti-JEV IgM antibodies in patient serum and CSF samples.

Type of sample	% Agreement[a]	% Sensitivity[b]	% Specificity[c]
Serum	98%(49/50)	96%(29/30)	100%(20/20)
CSF	96%(29/30)	95%(20/21)	100%(09/09)

[a](Number of samples positive by both method + number of samples negative by both methods) /(total number samples) x100.
[b]True positive/(true positive + false negative) x 100.
[c]True negative /(true negative + false positive) x 100.

Comparison of the in-house dipstick ELISA using rJEV EDIII protein with Pan-Bio IgM capture ELISA and JEV Chex IgM capture ELISA for detection of IgM antibodies to JE revealed comparable sensitivities, specificities and overall agreements (Table 4.5 and 4.6). The reaction pattern of in-house developed dipstick ELISA using rJEV EDIII protein with positive and negative serum and CSF samples are shown in figure 4.5.c. Prior to evaluation with patient sera, rJEV EDIII protein was checked using hyper immune sera against JE virus. Moreover rJEV EDIII protein has further been evaluated with six JE virus infected PCR positive samples (Parida et al., 2006). The rJEV EDIII protein recognized antibodies in JE virus infected and PCR positive samples thereby revealing its usefulness. To check non-specificity, this protein was tested with 20 serum samples collected from healthy person. None of these serum samples were, however, found to give positive reaction with this antigen and established its specificity for detection of JEV infection.

Table 4.6. Comparative evaluation of in-house ELISA with reference to JEV CheX IgM capture ELISA for detection of anti-JEV IgM antibodies in patient serum and CSF samples.

Type of sample	% Agreement	% Sensitivity	% Specificity
Serum	96%(48/50)	93%(29/31)	100%(19/19)
CSF	96%(29/30)	95%(20/21)	100% (09/09)

4.4 DISCUSSION

Laboratory scale expression of recombinant protein is generally performed using complex medium such as LB at shake flask culture. However, for producing protein in bioreactor, trial with different media is necessary. Composition of media seem to be important to ensure proper cell growth and for rJEV EDIII protein production. The present study therefore had an objective to produce rJEV EDIII protein with a high yield and evaluate its use in diagnosis of the disease as well as for vaccine development in future. The use of complex media as well as chemically defined media in producing recombinant proteins is a common practice (Lim and Jung, 1998; Kweon et al. 2001; Manderson et al., 2006; Tripathi et al., 2009). The complex media give more consistent yields, allow easier process control and simplify downstream recovery of the target protein. High-cell densities attained with SB medium can be explained, as this media is rich in yeast extract and phosphate salts as well as supplemented with glycerol compared to the other media used. Yeast extract is a known source of trace components and can relieve cellular stress responses such as the production of proteases during synthesis of recombinant protein. The phosphate salts in the media provided a buffering capacity to prevent pH fluctuations that could adversely affect normal metabolism (Lim et al., 2000).

Several recombinant proteins have been successfully produced in *E. coli* by fed-batch cultivation using various regimes of nutrient feeding resulting in different biomass and product yields (Lee, 1996; Babu et al., 2000; Khalilzadeh et al., 2004). To enhance the yield, rJEV EDIII protein was produced by batch and fed-batch cultivation processes. As the biomass is accumulated during the cultivation, the oxygen demand is also increased. Once feeding is initiated and *E. coli* enters into log phase, the feed is consumed more or less in an exponential manner. But, the feeding rate has to be controlled so that it does not exceed the

nutrient demand or feed consumption rate. It is done by maintaining the pH and DO around their set values. A fall in pH and DO is an indication of substrate overdosing. Rise in pH and DO values indicate that the carbon source or one of the substrate is limiting hence feed is required. The composition of the growth media is crucial for enhancing product formation as well as acetate reduction. Acetate is not produced when glycerol is used as the source of carbon, and the high cell densities with less foaming may be achieved relatively easily using glycerol. The lower rate of glycerol transport in the cell, compared with that of glucose, apparently leads to a reduction in the flux of carbon through glycolysis; greatly reducing acetate formation. *E. coli* is able to utilize acetate as a carbon source when glucose is absent. The consumption of acetate is characterized by a deviation from the preset values to lower pH values and cyclic patterns start appearing in the consumption of oxygen, till the preset pH value is gradually regained by the culture (Bhatnagar et al., 2008). At this time feeding is restarted. Phosphate is also known to be important for attaining high-cell densities of *E. coli*, as phosphate can easily become a limiting nutrient when provided in low doses (Korz et al. 1995). The feeding strategy used in the present experiments was a combination of both the pH-stat as well as DO-stat methods. Monitoring of both the parameters simultaneously gives better control over the growth conditions of the growing culture. The final dry cell weight after fed-batch cultivation (36.45 gl^{-1}) is about twenty eight times greater than that of shake flask culture (1.30 gl^{-1}) using LB medium and more than eight times when compared with batch cultivation (4.16 gl^{-1}) using SB medium.

Expression of heterologous protein in *E. coli* allows its rapid and economical production in large amounts. Efforts to obtain soluble target protein in host *E. coli* strain, inclusion body formation is still considered as a convenient and effective way in recombinant protein production. Expression of target protein (5-30%) of the total cellular protein, easy isolation of the inclusion bodies from cells, lower degradation of the expressed protein, high level of target protein homogeneity in inclusion bodies, and possibility to reduce the number of purification steps are usually indicated as the main advantages of IBs formation (Singh and Panda, 2005). In contrast to many reported protocols for the purification of recombinant viral envelope EDIII protein (Pattnaik et al., 2007; Verma et al., 2009), the present data demonstrate the feasibility of obtaining pure protein utilizing a single chromatographic step. More than 95% purity of protein as determined densitometrically from SDS-PAGE gels has

been achieved following a single step purification strategy which yielded ~910 mgl^{-1} of rJEV EDIII protein. Thus, fed-batch cultivation has led to about more than eight times purified protein yield in comparison with batch cultivation using SB medium.

The rJEV EDIII protein shows reactivity with the antibodies in JE virus infected patient serum and CSF samples. Thus this protein could be used in the diagnosis of JE infection. The diagnosis of JE has advanced considerably in recent years and routine laboratory diagnosis of JE virus infection is primarily carried out by detection of anti-JE virus antibody by serological methods. MAC-ELISA and indirect IgM ELISA is considered as a valuable diagnostic tool in JE virus infection (Bundo and Igarashi, 1985; Solomon et al., 1998; Ravi et al., 2006; Jacobson et al., 2007; Shrivastva et al., 2008; Ravi et al., 2009; Litzba et al., 2010).The Pan-Bio IgM capture ELISA (Pan-Bio, Australia) and JEV CheX IgM capture ELISA are commonly used test for JE virus infection. Both tests are based on the capture principle and JEV Chex kit (Ravi et al., 2006) uses native viral antigens (high production cost and produces biohazards). Pan-Bio JE-Dengue IgM combo ELISA is based on recombinant envelope protein that can be exploited for the detection of both Dengue and JE virus but showed cross-reactivity. Therefore, there is a genuine need for a promising test system for detection of JE infection.

In the present study, we have tested 80 specimens (50 serum and 30 CSF) for the presence of IgM antibodies by Pan-Bio IgM capture ELISA, JEV-CheX IgM capture ELISA and in-house ELISA test. The in-house indirect ELISA test has shown comparable sensitivity, specificity and more than 94% accordance for detection of anti-JEV IgM antibodies as compared to both commercial assays. The in-house dipstick ELISA is a qualitative test meant for field use. The common problem with JE serological assays lies in detection of circulating cross reactive antibodies against other members of *Flavivirus*. This cross reactivity could be found to be significantly reduced while identifying JE virus infection with ELISA employing pre-coated cell culture purified antigen as compared to mouse brain or crude cell culture antigen (Yang et al., 2006). The rJEV EDIII protein obviates expensive and time consuming cultivation of virus (for antigen preparation) and the associated biohazard risk. The present study demonstrates that the in-house developed ELISA test using rJEV EDIII protein can be used as a promising test in developing countries, which will help authorities to undertake effective control measures and adopt management

strategies against impeding JE menace. The domain III of the envelope protein of Japanese encephalitis virus is particularly an important antigen for vaccine development as well as its use as a reagent for diagnostic purposes**,** as it contains neutralizing epitopes and host cell receptor recognition (Kaur and Vrati, 2003; Wu et al., 2003; Alka et al., 2007; Shukla et al., 2009; Verma et al., 2009) site. Increase in the production of rJEV EDIII protein for application in diagnostic use as well as for further vaccine studies was necessary. Optimization of cultivation medium and cultivation conditions as described in this study, made its application more feasible. Further**,** the use of rJEV EDIII protein as an antigen in dipstick ELISA resulted in good agreement with the findings of commercial capture ELISA systems. These results show that the product has a promising potential for its use in diagnosis of JE both in laboratory and field condition with comparable sensitivity and specificity.

4.5 CONCLUSION

A simple fed-batch cultivation process has been demonstrated to produce rJEV EDIII protein in *E. coli* with a high yield in its biologically active form. *E. coli* is the most widely used organism for recombinant protein production because of its rapid growth to high-cell densities on inexpensive substrates and its well-characterized genetics and proteomics. Production of recombinant JE virus envelope domain III protein was influenced by media. Recombinant JEV EDIII protein, produced in super broth and terrific both media gave better growth and produces more protein in comparison with other media. For cost-effective reasons, it is important to maximize protein production by development of efficient cultivation process and appropriate purification methods. The fed-batch cultivation strategy employed in this study is probably one of the cost-effective means to enhance cell mass and proteins production. Taking advantage of high-volumetric yields obtained by fed-batch cultivation, ~910 mg of rJEV EDIII protein per liter of culture broth was produced. These result exhibit the ability to produce high yield of JE virus EDIII protein. Large quantity of antigenically active recombinant protein, produced by these methods may possibly be used for diagnosis as well as for further studies of immunoprophylaxis in JE virus infection. This production strategy can also be used for high yield production of other recombinant viral proteins.

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CHAPTER 5

DEVELOPMENT OF A PILOT SCALE PRODUCTION PROCESS FOR RECOMBINANT JEV EDIII PROTEIN AND CHARACTERIZATION FOR ITS VACCINE POTENTIAL

ABSTRACT

Japanese encephalitis (JE) virus is the most important cause of encephalitis in most of Asian regions. JE virus envelope domain III (JEV EDIII) protein is involved in binding to host receptors and it contains specific epitopes that elicit virus neutralizing antibodies. A highly immunogenic, recombinant JEV EDIII protein was expressed in *Escherichia coli*. In order to take this vaccine candidate for further studies, recombinant JEV EDIII protein was produced employing a pilot scale fermentation process. Recombinant JEV EDIII protein expressed as inclusion bodies was solubilized in 8 M urea and renatured by on-column refolding protocol in the presence of glycerol. Three step purification process comprising of affinity chromatography, ion-exchange chromatography (IEX) based on salt and IEX based on pH was developed. About ~124 mg of highly purified and biologically active EDIII protein was obtained from 100 g of biomass. Biological function of the purified EDIII protein was confirmed by their ability to generate EDIII specific antibodies in mice that could neutralize the virus. These findings suggest that recombinant JEV EDIII protein in combination with compatible adjuvant is highly immunogenic and elicit high titer neutralizing antibodies. Thus, recombinant JEV EDIII protein produced at large scale can be a potential vaccine candidate.

5.1 INTRODUCTION

Japanese encephalitis is the most important form of viral encephalitis in Eastern and Southern Asia and parts of Western Pacific (Liang et al, 2009; Fischer et al, 2010). There is no specific treatment for JE and hence vaccination of susceptible populations is the sole logical alternative. The vaccines that are available, namely the mouse brain or cell culture derived formalin inactivated JEV vaccines are inherent with certain drawbacks. Besides being expensive and in short supply, it causes allergic reactions in some recipients (Plesner, 2003). Moreover, the immunity conferred by the vaccine is of short-term duration (Poland et al., 1990). Hence, there is an urgent need to develop alternate vaccine candidates that are effective, safe as well as affordable (Bharati and Vrati, 2006). The envelope protein (53–55 kDa) is a typical membrane glycoprotein, forming the outer structural protein component of the virus, and is responsible for a number of important processes such as viral

attachment, fusion, penetration, virulence and attenuation (Sumiyoshi et al., 1987; Lindenbach and Rice, 2001; Witthajitsomboon et al., 2010). The envelope domain III protein is also the major antigen responsible for eliciting neutralizing antibodies that confer protection to the host (Wu et al., 2003; Alka et al, 2007). Thus, EDIII protein could be employed as an immunogen for developing a JEV subunit vaccine.

Currently, there is a need for the production of cost effective and safe recombinant EDIII protein for the development of protein subunit vaccines or diagnostic reagents. For vaccine purpose, the recombinant proteins produced must maintain their biological activity (i.e., generate neutralizing antibodies against wild-type virus or able to bind to anti-JEV antibodies found in patient serum). The recombinant E, NS1 or EDIII proteins may be expressed using various hosts, such as bacteria, yeast and tobacco plants (Lin et al., 2008; Shukla et al., 2009; Appaiahgari et al., 2009). *Escherichia coli* is by far the most commonly used host for the expression of recombinant viral proteins. Small scale expression is widely used for optimizing conditions for a large-scale production of recombinant proteins (Tripathi et al., 2009).

The scale up process of recombinant JEV EDIII protein production may be performed by replacing commonly used shake flasks (Alka et al., 2007; Lin et al., 2008; Shukla al., 2009) to fed-batch or batch fermentations (Tripathi et al., 2010). To facilitate the purification of recombinant proteins, the proteins are commonly produced as fusion proteins that comprise of the EDIII protein fused with an affinity tag, such as the hexahistidine tag (Wang et al., 2009; Shukla et al., 2009). High-level expression of recombinant proteins in *E. coli* often accumulates as insoluble aggregates in the form of inclusion bodies (Fahnert et al., 2004). To recover active protein, inclusion bodies must be solubilized and refolded. Chemical chaotropes have been traditionally used to solubilize proteins from inclusion bodies. High concentrations of chaotropes (such as up to 6 M guanidine hydrochloride or 8 M urea) are required to provide the chemical energy to thermodynamically dissociate the aggregates with concomitant denaturing of the protein. Reducing agents such as dithiothreitol (DTT) or β-mercaptoethanol are added to reduce all disulfide bonds (Wang et al., 2009).

Refolding is usually achieved by removing the chaotrope via buffer exchange after solubilizing the inclusion bodies, using dilution, dialysis, diafiltration in the presence of reduced glutathione and oxidized glutathione. However, refolding yields are typically low (Singh and Panda, 2005; Wang et al., 2009). Protein refolding by liquid chromatography is an alternative to the dilution refolding and has been put much highlight in recent years, and it was recently nominated protein folding liquid chromatography (Wang et al., 2009). Immobilized metal ion affinity chromatography (IMAC) has become a well-established and versatile technique for both analytical and large scale of protein separations. It has recently been reported that IMAC has the potential to perform protein refolding with high recovery of purified recombinant proteins (Wang et al., 2009). After the preliminary purification using affinity chromatography, the purity level of these proteins can be further enhanced by ion exchange or size exclusion chromatography (Mazumdar et al., 2010; Tan and Ng, 2010).

In previous chapter, we described the small scale production of rJEV EDIII protein from *E. coli* as well as affinity purification under denaturing conditions for use as diagnostic reagent (Tripathi et al., 2010). In the present study, we described here a scalable process for the production, refolding and purification of recombinant EDIII protein from *E. coli* for use as vaccine candidate. This process utilized a scale up of fermentation process and rJEV EDIII protein purification using affinity chromatography with simultaneous refolding, followed by ion exchange chromatography (IEX) based on salt and pH based ion exchange chromatography. Further, we present data demonstrating that the antibodies raised in mice against refolded and purified rJEV EDIII protein neutralize the JE virus. The rJEV EDIII protein was highly pure and found to be biologically active.

5.2 MATERIALS AND METHODS

The details about materials and methods used for pilot scale fermentation, on-column refolding, ion exchange chromatography, immunogenicity studies and plaque reduction neutralization test are described in the chapter 3. Figure 5.1 provides an overall flow diagram for the production of rJEV EDIII antigen for vaccine studies.

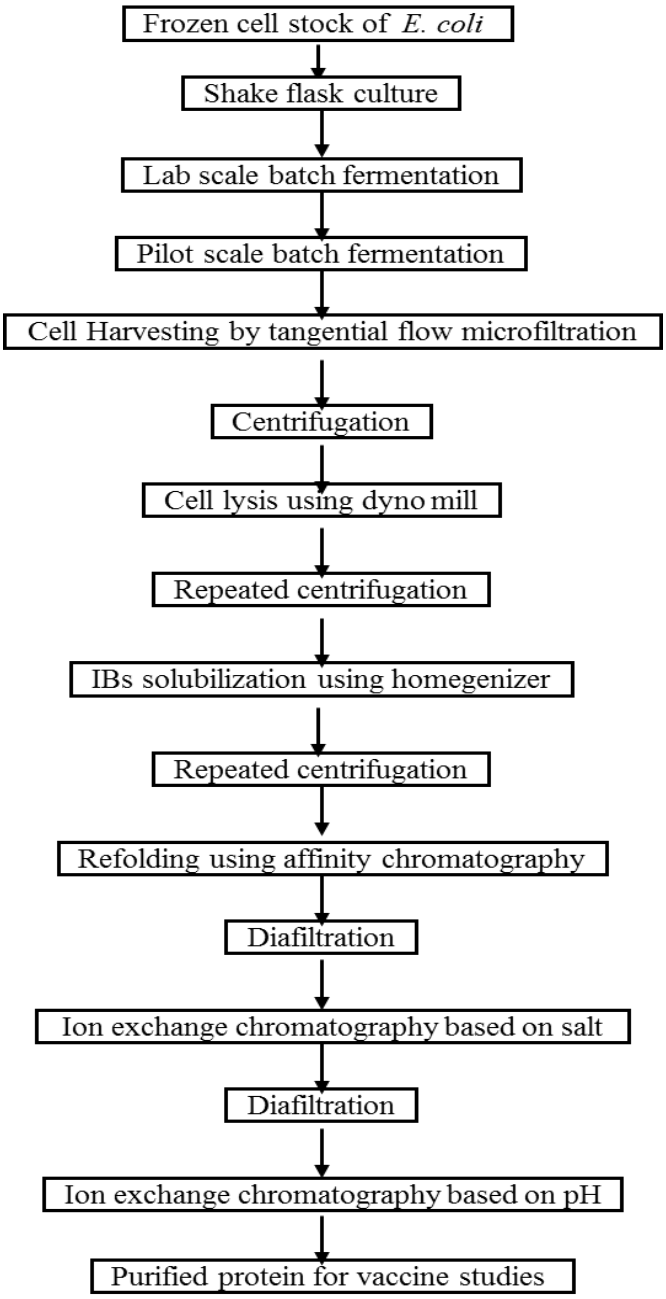

Figure 5.1. Process flow-sheet outline for production of rJEV EDIII protein.

5.3 RESULTS

Japanese encephalitis is a major cause of encephalitis in Asia. Cases occur largely in rural areas of the South and East Asian region resulting in significant morbidity and mortality. Multiple vaccines exist to control Japanese encephalitis, but all of them suffer

from problems. Envelope protein domain III of Japanese encephalitis virus is involved in binding to host receptors and it contains specific epitopes that elicit virus-neutralizing antibodies. To conduct vaccine studies with rJEV EDIII antigen, pure and biologically active protein was required. A recombinant clone of *E. coli* BL21 (DE3) cells was previously developed using the plasmid (pET 30a+rJEV EDIII) construct to produce this protein at small scale and using this clone we further developed a pilot scale fermentation process to produce rJEV EDIII protein in large quantities for use in vaccine studies.

5.3.1 Pilot Scale Production of recombinant JE virus EDIII protein

Pilot scale fermentations were carried out to produce large amount of biomass for purification of rJEV EDIII protein used in vaccine studies. Before the pilot scale fermentation, the fermentation conditions and culture medium were optimized at the small scale using shake flask culture and 5 liter fermentor. Under the fermentation conditions optimized at the small scale, production of rJEV EDIII protein was carried out using 100 liter working volume fermentor with 85 liter modified SB medium. During the cell growth period, pH was maintained around 7.0 by an automatic pH control system, and the cultivation temperature was 37°C; the air was supplied at the rate of 25-50 liter min^{-1} (LPM) and the dissolved oxygen level was not lower than 20%. The agitation speed, the flow rate of aeration, and supply of pure oxygen were adjusted to maintain the DO level above 20% air saturation. This fermentation process continued for nine and half hour which comprised of the pre-induction (five and half hour) and post induction (four hour) phase. At the beginning of the fermentation DO was above 30% and decreased during course of process. The DCW and OD_{600} increased continuously and reached a final value of 6.31 g l^{-1} (Table 5.1) and 19.80 (figure 5.2) respectively. The DCW at induction was 2.70 g l^{-1}. The DCW at induction and harvest after batch fermentation using 5 liter fermentor was 2.60 g l^{-1} and 6.22 g l^{-1} (Table 5.1) respectively. The successful production of this protein in fermentor with a high yield was initiated to foster further applications and research with JE vaccines. Reproducibility of all batch fermentations were confirmed with additional experiments conducted under the above specified optimal conditions and the final yield was within less than 10% of the result shown in table 5.1. The pilot scale fermentation yielded approximately 2 kg cell paste that was aliquoted and frozen at −80°C. The real time profile of a pilot scale batch fermentation process is shown in figure 5.2.

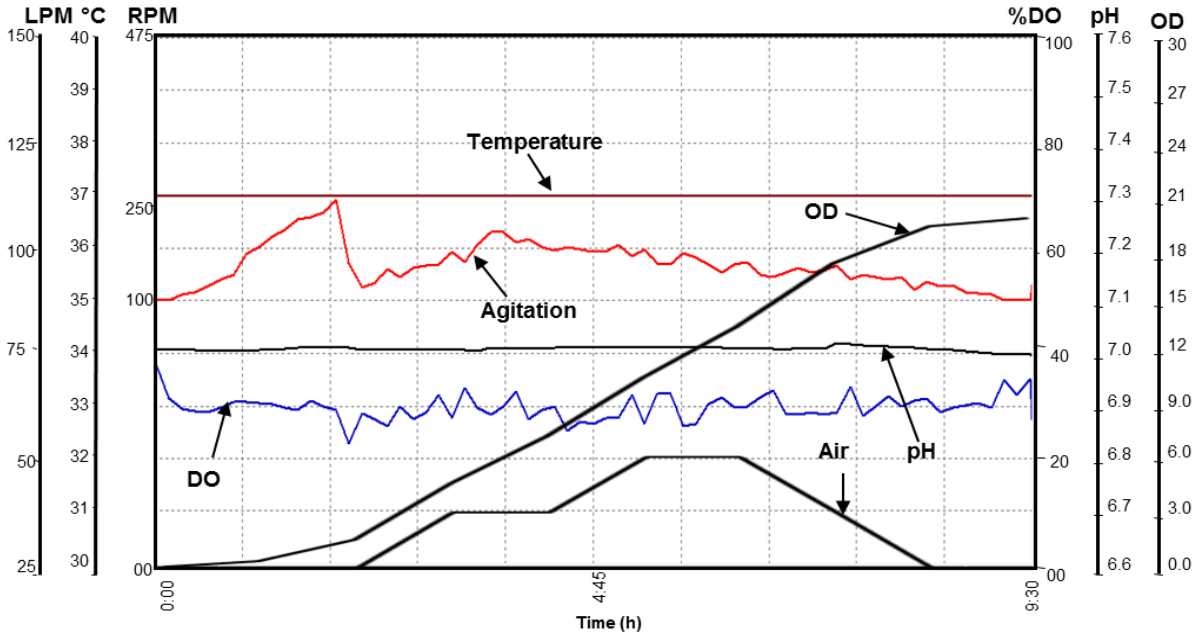

Figure 5.2. Real-time profile of pilot scale batch fermentation of E. coli BL21(DE3) [pET30a+rJEV EDIII gene] for the expression of rJEV EDIII protein. Culture was grown in batch mode in modified SB media and induced with 1 mM IPTG at OD_{600} of 10.8 (DCW = 2.7 g l^{-1}) for expression of rJEV EDIII protein. Figure shows time profile for agitation (rpm), dissolved oxygen concentration (%), air flow rate (liter min^{-1}), temperature (^{o}C), pH and OD_{600}.

Table 5.1. Production characteristics of *E. coli* expressing rJEV EDIII protein.

Media type	Cultivation mode	Dry cell weight (DCW) (g l^{-1})	Specific product yield (mg g^{-1} DCW)	Final product concentration[c] (mg l^{-1})
SB	Shake flask	2.15	11.31	24.32
Modified SB	Shake flask	2.64	16.15	42.66
Modified SB	Batch fermentation[a]	6.22	20.93	130.24
Modified SB	Batch fermentation[b]	6.31	21.36	134.83

[a]At 5.0 liter scale; [b]At 100 liter scale; [c]After on-column refolding with affinity purification.

5.3.2 Refolding and purification of rJEV EDIII protein

We developed purification process for recombinant Japanese encephalitis envelope domain III protein to obtain a high quality protein suitable for human Japanese encephalitis vaccine studies. Purification of rJEV EDIII protein was performed from the cell

pellet obtained from fermentation. After cell disruption and centrifugation, analysis of the lysate confirmed the presence of the major proportion of ~13 kDa protein band. Inclusion bodies were harvested and purified from the induced and lysed cell mass. On-column refolding was carried out using immobilized metal affinity chromatography. The final product concentration of rJEV EDIII protein following IMAC was significantly higher for pilot scale batch fermentation as compared to shake flask culture. Improvement in DCW (2.5-fold higher) and product yield (5-fold higher) was obtained for both lab and pilot scale batch fermentation using modified SB medium as compared to shake flask culture using SB medium (Table 5.1). Chromatogram of simultaneous refolding and purification of rJEV EDIII protein using IMAC is shown in figure 5.3.

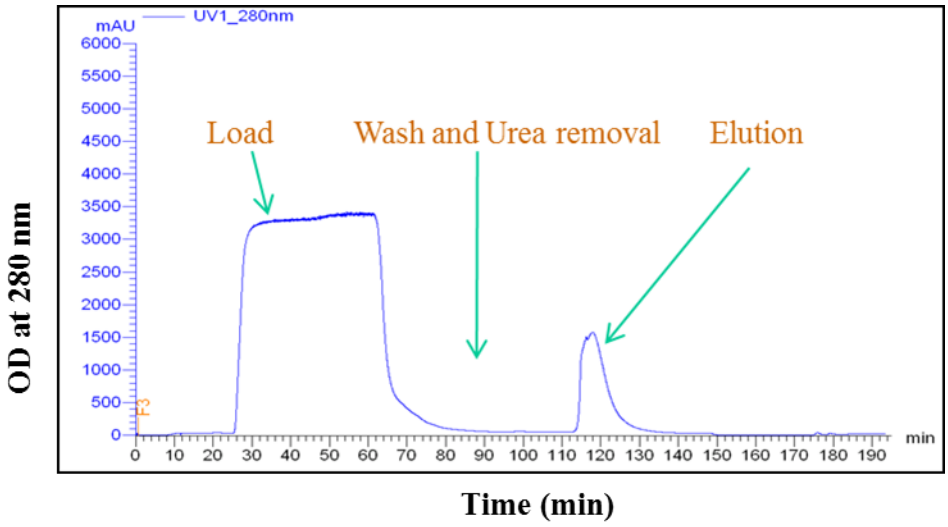

Figure 5.3. Chromatogram showing simultaneous refolding and affinity chromatography for rJEV EDIII protein.The solubilized IBs containing rJEV EDIII protein was loaded onto a 70/55 Quick scale chromatography column containing 500 ml Ni charged streamline chelating. Unbound proteins were removed by washing the column with five column volume of the buffer containing 50 mM imidazole and the refolded rJEV EDIII protein was eluted with buffer containing 300 mM imidazole.

Further, rJEV EDIII protein was purified to improve purity using ion exchange chromatography. Processing of biomass from pilot scale batch fermentation yielded ~124 mg of highly pure, refolded rJEV EDIII protein from 100 g wet biomass. The rJEV EDIII protein yield after different purification steps is given in table 5.1 and 5.2.

Table 5.2. Purification profile of rJEV EDIII protein purification process from 100 g biomass (cell pellet).

Purification process	Amount of protein[a] (mg)	Purity (%)	Overall yield (%)
Solubilizing of inclusion bodies	1869.3	60	100
Refolding and IMAC	606.8	91	32.4
IEX on SP-sepharose salt based	191.1	97	10.2
IEX on SP-sepharose pH based	124.2	99	6.6

[a]Amount of protein is given after chromatographic step

5.3.3 Characterization of rJEV EDIII Protein

Pilot scale produced and purified rJEV EDIII protein was characterized for its purity and reactivity using SDS-PAGE and Western blot analysis respectively. SDS-PAGE analysis of rJEV EDIII protein showed single band corresponding to ~13 kDa (Figure 5.4, 5.5.a).

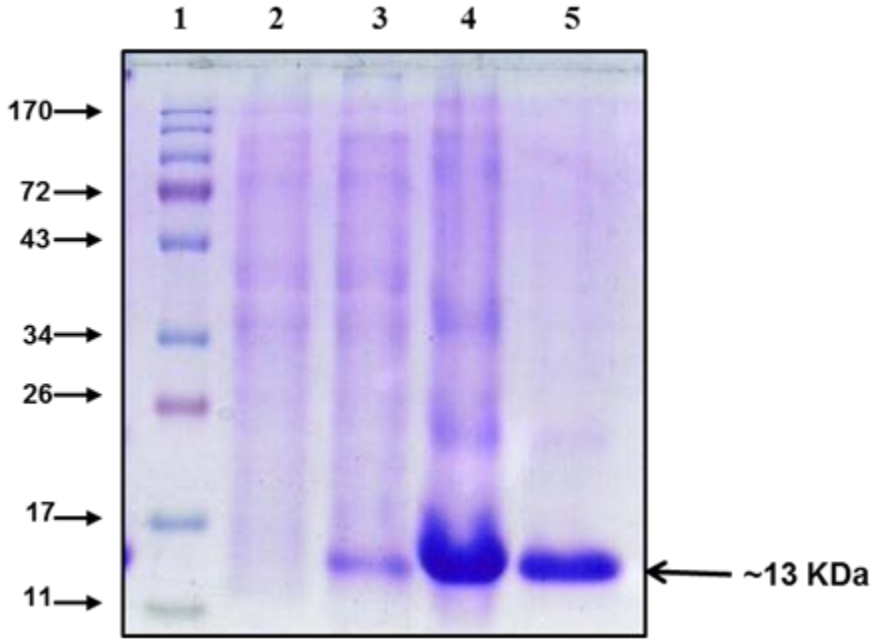

Figure 5.4. Coomassie blue stained SDS-PAGE. The protein band at approximate molecular weight of ~13.0 kDa confirmed the predicted size of the recombinant JEV EDIII protein. The protein profiles of the eluted protein and solubilized IBs in gel were analyzed densitometrically using Quantity One image quantification software which showed that more than 95% purity had been achieved. Lane 1, Molecular Weight Marker (kDa); lane 2,

Uninduced culture; lane 3, Induced culture, lane 4, Solubilized IBs; lane 5, on-column refolded and IMAC purified rJEV EDIII protein (10 µg).

A densitometry analysis of the protein bands of eluted protein and solubilized IBs in SDS-PAGE gel (Figure 5.4, 5.5.a) using Quantity One image quantification software (Bio-Rad, USA) clearly indicates greater than 98% purity. The western blot with hyper immune sera raised in mice revealed that the sera reacted with rJEV EDIII protein (Figure 5.5.b) and confirmed the usefulness of this protein. The endotoxin content of purified protein was less than 5 endotoxin unit (EU) per 25 µg of rJEV-DIII antigen used for immunization.

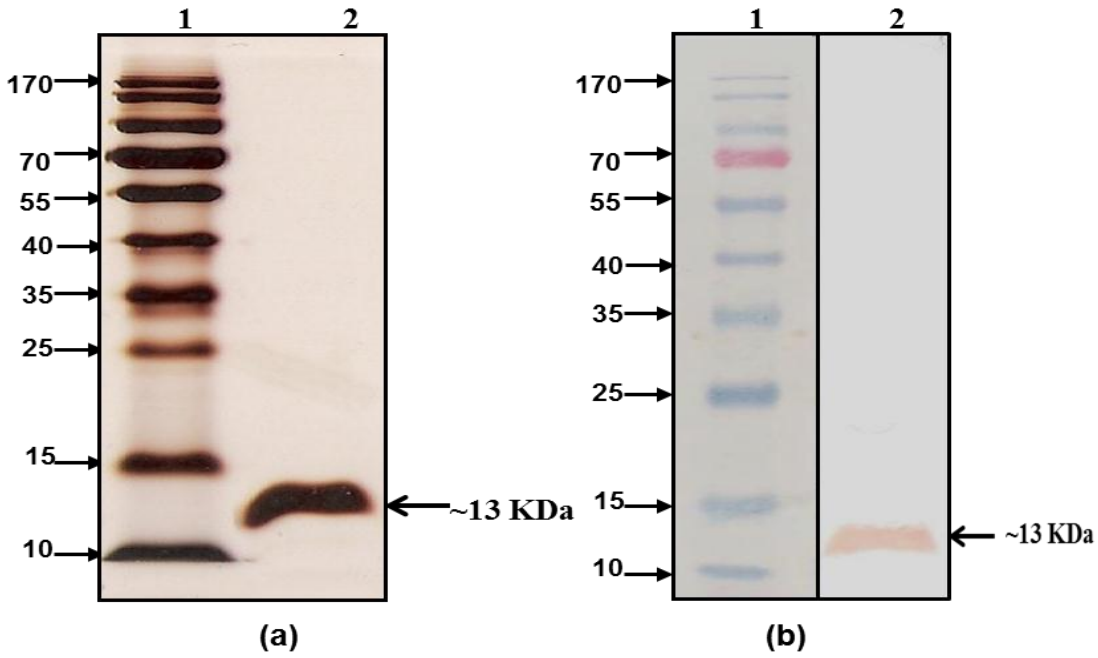

Figure 5.5. Characterization of pH based IEX purified rJEV EDIII protein produced at 100 liter scale. (a) Purity of rJEV EDIII was assessed on SDS-PAGE gel after silver staining. Lane 1, Molecular Weight Marker (kDa); lane 2, Purified rJEV EDIII protein (10 µg). (b) Western blot analysis of rJEV EDIII protein with polyclonal sera raised against rJEV EDIII protein in mice. Lane 1, Molecular weight marker; lane 2, Purified rJEV EDIII protein.

5.3.4 Humoral Response in Mice Immunized with rJEV EDIII Protein

Refolded, purified and characterized recombinant JEV EDIII protein was formulated with FCA adjuvant and used to immunize six BALB/c mice (25 µg rJEV EDIII protein per dose). The control group, which received adjuvant alone, was also comprised of six mice. A

final bleed was collected two weeks after second boost. Sera collected from mice were serially diluted and tested for recognition of rJEV EDIII protein by ELISA. Adjuvant control sera tested at 1:1000 dilution yielded absorbance value at 490 nm (A490) which was used as negative control. The A490 values with sera from mice immunized with FCA are clearly higher (Figure 5.6). The rJEV EDIII antigen specific antibody titer was found to be more than 2, 56,000 (Figure 5.6). The result shows that rJEV EDIII expressed in *E. coli* is able to elicit EDIII-specific antibody responses. Absorbance of control mice sera in 1:1000 dilution was 0.720. The end point titers of the FCA group and control sera are shown in figure 5.6. The rJEV EDIII antigen with FCA was highly immunogenic and yielded good ELISA titers.

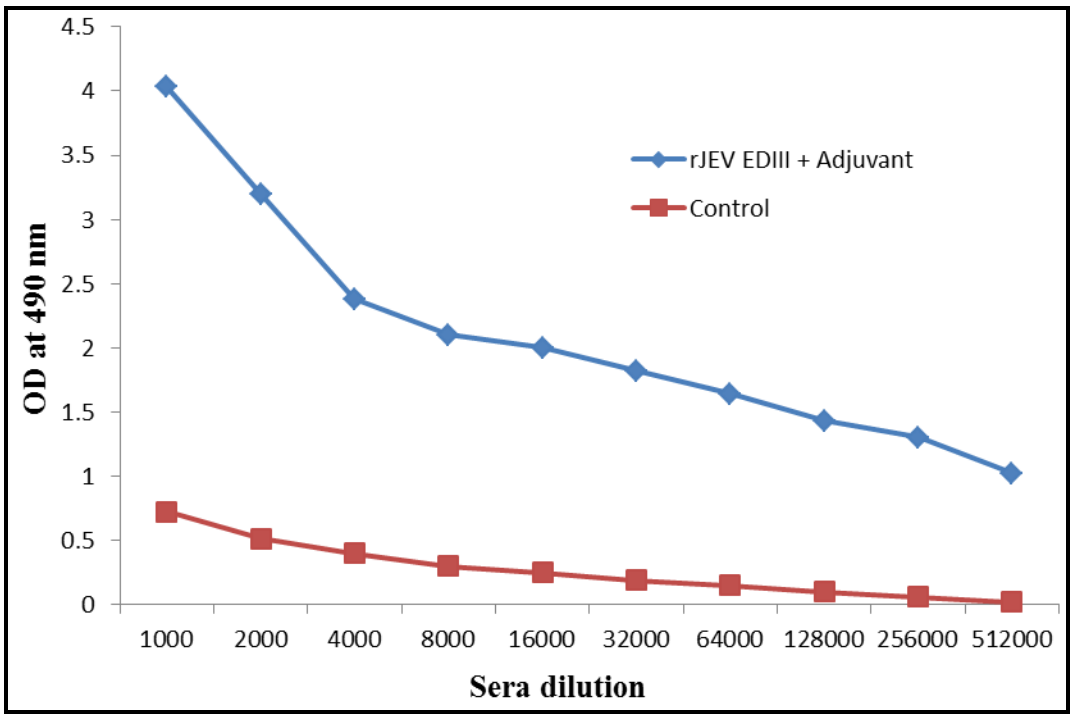

Figure 5.6. Endpoint ELISA titers for recognition of rJEV EDIII protein. Sera from mice collected at days 56 were tested for recognition of recombinant JEV EDIII at various dilutions by ELISA.

5.3.5 Plaque Reduction Neutralization Test

The neutralization effect of polyclonal antibodies raised against recombinant JEV EDIII protein on the Japanese encephalitis virus was evaluated by plaque reduction neutralization test in Vero cells. Two-fold serum dilutions were tested starting at 1:2 against

100 p.f.u. of JE virus. $PRNT_{90}$ values of JE virus by anti-rJEV EDIII antibodies was observed up to a dilution of 1:64 (Figure 5.7). $PRNT_{90}$ titers of the rJEV EDIII anti-serum were approximately 1:32 for FCA group sera. Complete neutralization of JE virus by anti-EDIII antibodies was observed up to a dilution of around 1:16 and high neutralization effect remained up to a dilution of 1:64 (Figure 5.7).

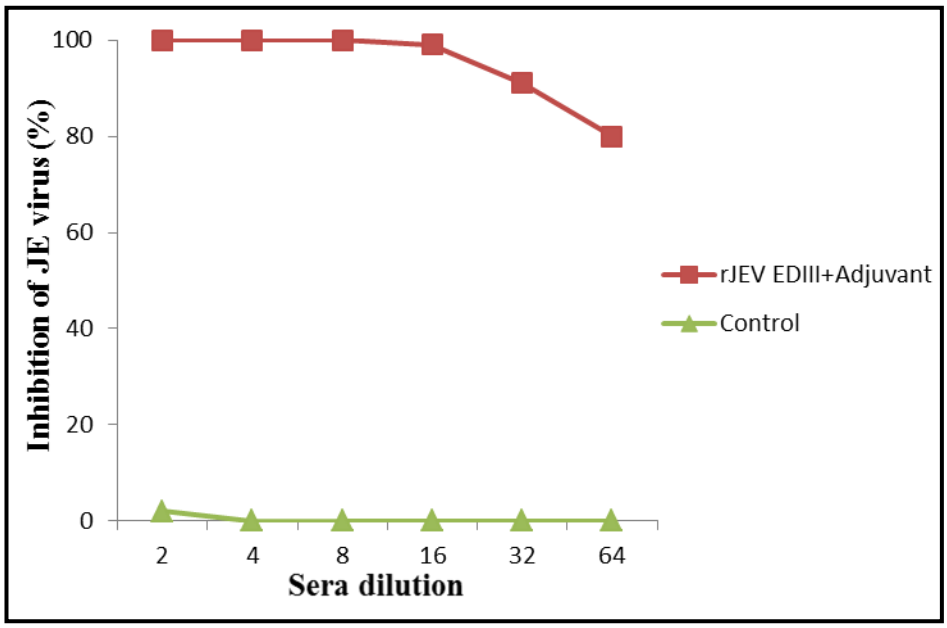

Figure 5.7. Neutralizing ability of sera generated against the recombinant JEV EDIII protein in mice formulated with Freund's adjuvant. Plaque reduction neutralization test was carried out with JE virus. Complete neutralization was achieved till 1:16 dilution and high neutralizing antibody titers remained till 1:64 dilution.

5.4 DISCUSSION

Selection of an antigen is an important aspect for the development of a vaccine. The envelope protein of Japanese encephalitis virus is the major antigen used to elicit neutralizing antibody response and protective immunity in hosts. In the previous studies, investigators have expressed the EDIII protein of JEV at shake flask culture in *E. coli* and evaluated the protection using these proteins (Wu et al., 2003; Alka et al., 2007; Lin et al., 2008; Verma et al., 2009). Production of important foreign proteins in *E. coli* expression system has been highly successful in recent past as this system offers several advantages over the other expression systems, including its fast growth rate, utilization of cheap growth media, high level of expression of foreign proteins (Yee and Blanch, 1993; Khalilzadeh et al., 2008;

Mazumdar et al., 2010) as well as simple and easy purification (Singh and Panda, 2005; Bhuvanesh et al., 2010). Therefore it is imperative to develop an efficient and scalable production and purification process. To enhance the expression of rJEV EDIII protein in *E. coli*, super broth and modified super broth media were tested. Modified SB medium resulted in more biomass and protein yield. Thus, modified SB medium was used for further studies. High-cell densities attained with modified SB medium (Table 5.1) can be explained, as this media is rich in glycerol as compared with SB media.

Scaling up production of an interesting recombinant protein requires both increasing the productivity and the expression level of the target protein, which are strongly influenced by fermentation conditions (Bell et al., 2009). The other critical factor for the development of any vaccine is the ability to produce the vaccine with a process that is amenable to scale-up, is reproducible and produces a reasonable number of doses of the vaccine. According to BC Buckland "Process development is the technological foundation that underlies the manufacture of new vaccines and is central to successful commercialization" (Buckland, 2005). Based on the earlier studies on rJEV EDIII protein, we envisaged to produce this vaccine candidate on large scale using pilot scale fermentor. In this study, we describe the process development by fermentation, purification and immunogenicity of a candidate vaccine recombinant rJEV EDIII antigen. Before scale-up, it is essential to ensure the reproducibility of the process to ensure its robustness. We have performed several fermentations and purifications to ensure that the process was easily scalable from the laboratory setup to the pilot scale production. Pilot scale fermentation was carried out using modified SB medium. Modified SB medium mainly comprised of yeast extract, phosphate salts and glycerol. Yeast extract present in growth media has been reported to enhance the specific cellular yield of the expressed protein particularly during high cell density cultivation where the demand of nitrogenous source becomes very high following induction (Manderson et al., 2006; Tripathi et al., 2009; Mazumdar et al., 2010). Glycerol is one of the commonly used carbon source. By using glycerol, high dry cell weight and cell densities may be achieved with less frothing (Khamduang et al., 2009). The phosphate salts in the media provided a buffering capacity to prevent pH fluctuations that could adversely affect normal metabolism (Lim et al., 2000; Babaeipour et al., 2010).

We developed purification process for rJEV EDIII protein to obtain a high quality

protein suitable for human JE vaccine studies. The initial step of purification with simultaneous refolding using immobilized metal affinity chromatography (IMAC) removed a majority of contaminants as well as denaturants. To refold the bound rJEV EDIII protein, the column with bound proteins was washed with the refolding buffer B which eliminated most of the contaminants in the flowthrough, and the urea concentration in the IMAC column was decreased to initiate the refolding of the denatured rJEV EDIII protein to its native state. The refolded rJEV EDIII protein was eluted with increased concentration of imidazole in the elution buffer. Therefore, rJEV EDIII protein can be refolded and purified simultaneously in one IMAC run. It was reported that a suitable concentration of urea (3 M) and glycerol (12%) in mobile phase is beneficial to protein refolding due to increased renaturation yield, protein stability and reduction in the formation of aggregates in liquid chromatography (Wang et al., 2009). To further purify rJEV EDIII protein, two-step process comprising of ion-exchange chromatography based on salt and pH was developed. Because the refolding of proteins is in competition with its aggregation, it is of great importance to find effective step for target protein monomer separation from dimer and other oligomers (Sereikaite et al., 2008). However, there was a drop in the yield of rJEV EDIII protein following ion exchange chromatography using salt and pH. We predict that this loss in the yield of rJEV EDIII following ion exchange chromatography was due to formation of multiple conformers of rJEV EDIII protein in *E. coli* having different surface charge properties, which may not be distinguishable on SDS-PAGE gel. Further attempt to purify single conformer of rJEV EDIII protein based on their surface charge property by ion exchange chromatography resulted in lower yield than OCR-AC. This observation also indicates that the higher yield of rJEV EDIII protein at affinity purification with simultaneous refolding stage may not necessarily lead to higher yield of purified rJEV EDIII protein following subsequent purification step as affinity chromatography will purify all conformers of rJEV EDIII protein produced in *E. coli* through histidine tag. About 124 mg of highly pure, refolded and biologically active rJEV EDIII protein was obtained form 100 g of pilot scale produced biomass. Though this yield is reasonable to proceed for production of this cGMP grade protein for use in human vaccine trials, efforts are being made to further improve the final yield. The subsequent characterization of recombinant JEV EDIII protein by SDS-PAGE, Western blot and ELISA confirms that rJEV EDIII protein is highly pure and reactive. It is evident from

the results that the rJEV EDIII protein specifically reacted with anti-JE virus antibody and thus suggests that this protein could also be used for the purpose of diagnosis of JE virus infection.

We have also studied the immunogenicity of recombinant JEV EDIII formulated with FCA adjuvant in BALB/c mice. When the antigen is small molecule or poorly immunogenic, the immune system requires a stimulus to induce an effective immune response. Adjuvants can be used for this purpose, and can direct an immune response towards a more cellular or humoral response (Cox and Coulter, 1997). FCA is an important adjuvant for investigators and is regarded scientifically as an effective means of potentiating immune responses in laboratory animals. Though, Freunds adjuvant is not approved for human use, the study further needs to be performed with human compatible adjuvants i.e., aluminium hydroxide gel, liposome based adjuvants or novel nanoparticle (Huang et al., 2010) based adjuvants. There is no universal adjuvant and it must be adapted according to several criteria in order to have the best balance between safety and efficacy. Our initial evaluations indicated that the rJEV EDIII protein in combination with adjuvant elicited humoral immune response in mice. The humoral response was characterized by high titers of antibodies as well as high neutralizing titers by plaque reduction neutralization assay. Neutralizing antibodies can bind to virus and prevent virus from binding to host cell receptors. The polyclonal antibodies raised in BALB/c mice using rJEV EDIII protein were found to be neutralizing JE virus entry in to Vero cells. This implies that the refolding of protein could correctly present the conformational epitopes on the surface of the recombinant JEV EDIII protein. FCA group showed virus neutralization at dilution of 1:32 and this corresponds to the high antibody end point titers with FCA adjuvant. The $PRNT_{50}$ titers $\geq$1:10 are considered indicative of protective immunity (Lin et al., 2008). The present study clearly reveals the potential of recombinant JEV EDIII protein to elicit neutralizing, and therefore, presumably protective antibodies against JE virus. The $PRNT_{50}$ titers reported recently using domain III based proteins were approximately 1:24 (Wu et al., 2003), 1:54 (Alka et al., 2007) and 1:28 (Lin et al., 2008). Hence, an ideal subunit vaccine using envelope protein needs to be correctly folded and should be well characterized. This is the first report on the use of a pilot scale produced, refolded and purified as well as fully characterized domain III protein which has

been used for the immunomodulatory studies in combination with adjuvant and detailed evaluation of humoral immune response.

5.5 CONCLUSIONS

In conclusion, we have developed a 100 liter pilot scale fermentation process for high yield production of recombinant Japanese encephalitis virus envelope domain III protein from *E. coli*. A simple, three-step purification strategy involving immobilized metal affinity chromatography with simultaneous refolding, followed by salt based ion exchange and pH based ion exchange chromatography, was used to purify rJEV EDIII protein that was highly pure, homogeneous and recognized by anti-JEV antibodies. Further, characterization for its immunogenicity and ability to induce neutralizing antibodies established its application more attractive. These findings suggest that rJEV EDIII protein in combination with compatible adjuvant is highly immunogenic and can elicit high titer neutralizing antibodies, which proves that refolded and purified rJEV EDIII protein can be a potential vaccine candidate. The method described here to produce rJEV EDIII protein may also be useful in producing other viral and bacterial proteins in *E. coli*.

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CHAPTER 6

PRODUCTION, PURIFICATION AND DIAGNOSTIC POTENTIAL OF RECOMBINANT JE VIRUS NONSTRUCTURAL 1 (NS1) PROTEIN

ABSTRACT

Japanese encephalitis (JE) is a major public health problem in South-East Asia and Western Pacific countries. The recombinant nonstructural 1 (rNS1) protein of JE virus is a potential diagnostic as well as vaccine candidate. Developments of cost effective and simple culture media as well as appropriate culture conditions are generally favorable for large scale production of recombinant proteins. The effects of medium composition and cultivation conditions on the production of rNS1 protein were investigated in shake flask culture as well as batch cultivation of *Escherichia coli*. Further, the fed-batch process was also carried out for high cell density cultivation (HCDC) of *E. coli* expressing rNS1 protein. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used to induce the expression of rNS1 protein at ~13 g dry cell weight (DCW) per liter of culture. The final DCW after fed-batch cultivation was ~17 g/l. The Inclusion bodies were isolated and purified through affinity chromatography to give a final product yield of ~142 mg/l. The reactivity of purified protein was confirmed by Western blotting and Enzyme linked immunosorbent assay. These results show that rNS1 protein may be used as a diagnostic reagent or for further prophylactic studies. This approach of producing rNS1 protein in *E. coli* with high yield may also offers promising method for production of other viral recombinant proteins.

6.1 INTRODUCTION

Japanese encephalitis virus (JEV) is the most important cause of epidemic encephalitis in most Asian regions with about 35,000-50,000 cases and 10,000 deaths annually (Solomon and Vaughn, 2002; Abraham et al., 2011). The Envelope (E) protein and nonstructural 1 (NS1) protein (Hua et al., 2010) elicits neutralizing antibodies and plays an important role in inducing immunologic responses in the infected host (Xu et al., 2004; Lin et al., 2008; Appaiahgari et al., 2009; Wang et al., 2009; Witthajitsomboon et al., 2010). NS1 specific antibodies have been demonstrated to provide protective immunity against dengue viruses (Amorim et al., 2010) and JE virus (Lin et al., 1998; Xu et al., 2004; Lin et al., 2008). In addition, the high immunogenicity of the NS1 proteins of JE, Dengue and other flaviviruses has raised considerable interest both as an antigen for diagnostic methods (Konishi et al., 2004; Lin et al., 1998; Huang et al., 2001; Xu et al., 2004; Konishi et al., 2009) and as component of subunit vaccine formulations (Lin et al., 1998; Xu et al., 2004;

Lin et al., 2008;). For this reason, the NS1 protein is an important immunogen for a subunit vaccine and also a prospective diagnostic reagent for the improved clinical diagnosis of JE virus infections.

There is no specific therapy for JE and vaccination is the only available preventive measure in addition to mosquito vector control. The internationally licensed vaccine has several limitations in terms of cost, availability and safety, apart from ethical issues (Plesner and Ronne, 1997; Appaiahgari et al., 2009). Early diagnosis of disease plays an important role to forecast an early warning of epidemic and to undertake effective vector control measures. The early diagnosis of JEV infection is achieved by serodiagnosis using ELISA based on the identification of NS1 antigen (Konishi et al., 2004; 2009) or anti-JEV IgM antibodies (Ravi et al., 2006; Jacobson et al., 2007; Shrivastva et al., 2008; Ravi et al., 2009; Litzba et al., 2010; Tripathi et al., 2010). Some of ELISA tests for detection of anti-JEV IgM antibodies utilizes whole-virus antigen prepared from cell culture so it is costly and also associated with biohazard risk. Replacement of the whole-virus antigens with recombinant envelope (E) protein eliminated the bio-safety risk but not the cross-reactivity problem (Ravi et al., 2006). However, all these kits are expensive due to the high costs associated with antigen production, making them unaffordable for use in the developing countries where JE is mostly prevalent. Thus, there is a need to develop detection system as well as an improved JE vaccine that may be safer, cheaper and readily available. Hence, production and purification of this protein is necessary for further studies. *E. coli* is the most commonly used host for heterologous protein production because it is a well-characterized organism in the genetics, physiology and cultivation condition (Lim et al., 2000; Khalilzadeh et al., 2008).

Protein expression level depends on cultivation conditions, such as medium composition, induction time, inducer concentration and inducer type, which can be optimized for over-expression of a recombinant protein (Manderson et al., 2006; Tripathi et al., 2009). Recombinant *E. coli* can be grown to high densities in complex media, semi-defined and defined media (Manderson et al., 2006; Khalilzadeh et al., 2008; Tripathi et al., 2011). The composition of the growth media is crucial for enhancing product formation as well as reduction of inhibitory compound formation (Manderson et al., 2006; Tripathi et al., 2009). Furthermore, one of the most popular methods to achieve high cell density is fed-batch culture by controlling the nutrient feeding via pH, dissolved oxygen (DO) or specific growth

rate (Lim et al., 2000; Manderson et al., 2006; Khalilzadeh et al., 2008; Bhuvanesh et al., 2010). Recombinant protein purification using the minimum possible steps is crucial to meet the required level of purity. Affinity chromatography is versatile and can be used for improved purification of recombinant proteins (Tripathi et al., 2010; Bhuvanesh et al., 2010).

There is currently a need for developing cultivation process for high yield production of recombinant NS1 protein of JE virus and development of cost-effective, safe and simple diagnostics. Thus, we have focused on the production of rJEV NS1 protein from *E. coli* which over-expressed this protein in the form of insoluble inclusion bodies. The culture media and culture conditions optimization; batch and fed-batch cultivation were performed to maximize overall productivity of this protein. Further, characterization of this protein was carried out for its potential as a diagnostic tool using ELISA makes its application more feasible.

6.2 MATERIALS AND METHODS

The materials used for this study are described in chapter 3. The methodologies used for this study are described in materials and methods section (chapter 3).

6.3 RESULTS AND DISCUSSION

6.3.1 Expression of Recombinant JE Virus NS1 Protein

The JE virus as well as dengue virus NS1 protein is a potential candidate for the design of subunit vaccines as well as diagnostic methods. Nonetheless, generation of recombinant (r) NS1 protein of dengue virus from infected tissue culture insect cells is a laborious and costly, subjected to batch-to-batch variation making it difficult for routine large-scale production (Huang et al., 2001). Expression of rNS1 protein of dengue virus in *E. coli* is a much cheaper and a simpler procedure (Huang et al., 2001; Das et al., 2009). In the present study, rJEV NS1 protein is produced in *E. coli* and used as a diagnostic reagent for detection of antibodies. The JEV NS1 coding sequence was previously cloned and, transformation of the *E. coli* SG13009 strain was carried out. The expressed proteins, following 4 h incubation in the presence of 1m M IPTG, were monitored by SDS-PAGE (Figure 6.1). These protein bands with molecular mass of ~44 kDa in the insoluble protein extracts of the recombinant strain corresponded to the predicted mass of JEV NS1 protein.

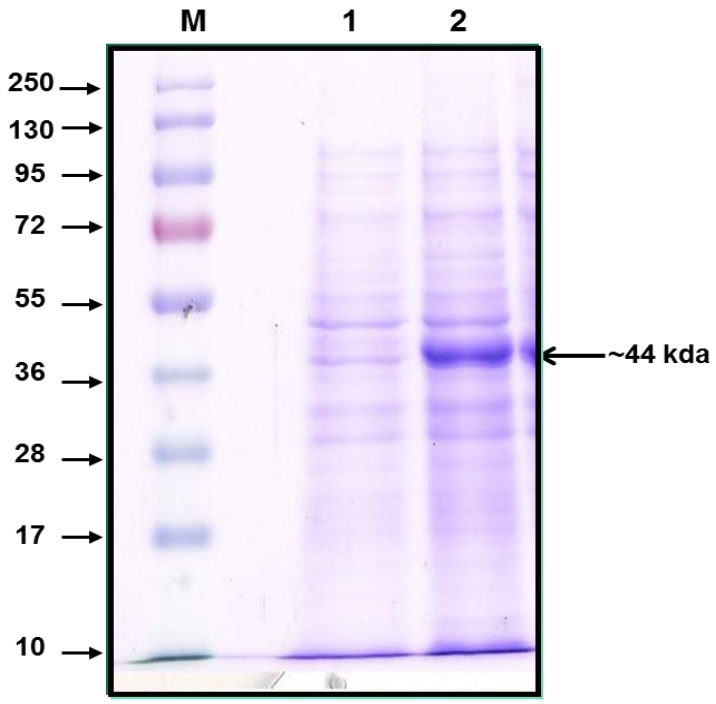

Figure 6.1. SDS–PAGE analysis of rJEV NS1 protein expression. The culture was induced with 1 mM IPTG and further grown for 4 h. Lane M, Molecular weight marker (kDa); lane 1, Un-induced coomassie stained E. coli lysates; lane 2, Induced culture lysates.

The appropriate medium for rJEV NS1 protein production was analysed by using shake flask experiments. For this purpose, four different media were tested. Maximum protein yield was obtained in modified SB medium followed by super broth medium. However, defined medium produced the lowest final cell density. Chemically defined media are generally known to produce slower growth and protein titers than semi-defined or complex media (Bhuvanesh et al., 2010). Thus modified SB medium was considered for further studies. The rJEV NS1 protein yield in different media are shown in table 6.1.

Table 6.1. Production characteristics of rJEV NS1 protein expressed in *E. coli* using different media in shake flask culture.

Media	DCW (g/l)	rJEV NS1 Protein (mg/l)
LB Broth	1.18	8.84
Super broth (SB)	1.77	13.02
Modified SB medium	2.14	16.10
Defined medium	1.05	6.69

6.3.2 Batch and Fed-batch Fermentation to Produce rJEV NS1 Protein

For *E. coli* or any other cultivation systems, the level of intracellular accumulation of a recombinant protein is dependent on the final cell density. Several recombinant proteins have been successfully produced in recombinant *E. coli* by fed-batch cultivation using various regimes of nutrient feeding resulting in different biomass and production yields. The development of a fed-batch process for high yield production of rJEV NS1 protein is required for further studies as a diagnostic reagent or prophylactic purpose. Batch cultivations with SB medium and modified SB medium were carried out. The DCW at the time of induction (after 5 h of cultivation) was 2.80 and 3.10 g/l respectively. The final DCW (~9 h of growth) at harvest in all media is given in table 6.2. The modified SB medium again resulted in more DCW and rJEV NS1 protein in comparison with SB medium (Table 6.2). This may be due to the presence of more glycerol in comparison with other media.

Table 6.2. Production characteristics of rJEV NS1 protein expressed in *E. coli* using different media in bioreactor.

Media	Culture condition	DCW (g/l)	rJEV NS1 Protein (mg/l)
Super broth (SB)	Batch cultivation	4.30	32.75
Modified SB medium	Batch cultivation	6.25	48.42
Modified SB medium	Fed-batch cultivation	17.78	142.16

This has already been established in earlier findings where yeast extract or glycerol was used as media components (Manderson et al., 2006; Khamduang et al., 2009). For further increasing the production of rJEV NS1 protein per unit volume, fed-batch cultivation using modified SB medium was carried out. The inducer (1 mM IPTG) was added to the culture at DCW of about 13.41 g/l and allowed to grow for another 4 h before harvesting. The growth profile (OD vs. Time) during course of fed-batch cultivation is shown in figure 6.2. The control of feeding rate is maintained by keeping the DO and pH values at their set point. Yeast extract present in growth media have also been reported to enhance the specific cellular yield of the expressed protein particularly during high cell density cultivation where the demand of nitrogenous source becomes very high following induction (Tripathi et al., 2009). Glycerol is one of the commonly used carbon source. By using glycerol, high DCW

and cell densities may be achieved with less frothing. The final DCW of about 17.78 g/l was obtained at ~13 h of growth in fed-batch mode (Table 6.2). The variation of cultivation parameters with time during fed-batch process is shown in figure 6.2. The DCW and rJEV NS1 protein yield in batch and fed-batch process with different media are given in table 6.2. The final DCW after fed-batch process was found to be increased more than fifteen times when compared with that of shake flask culture with LB medium and about ten times more with SB medium.

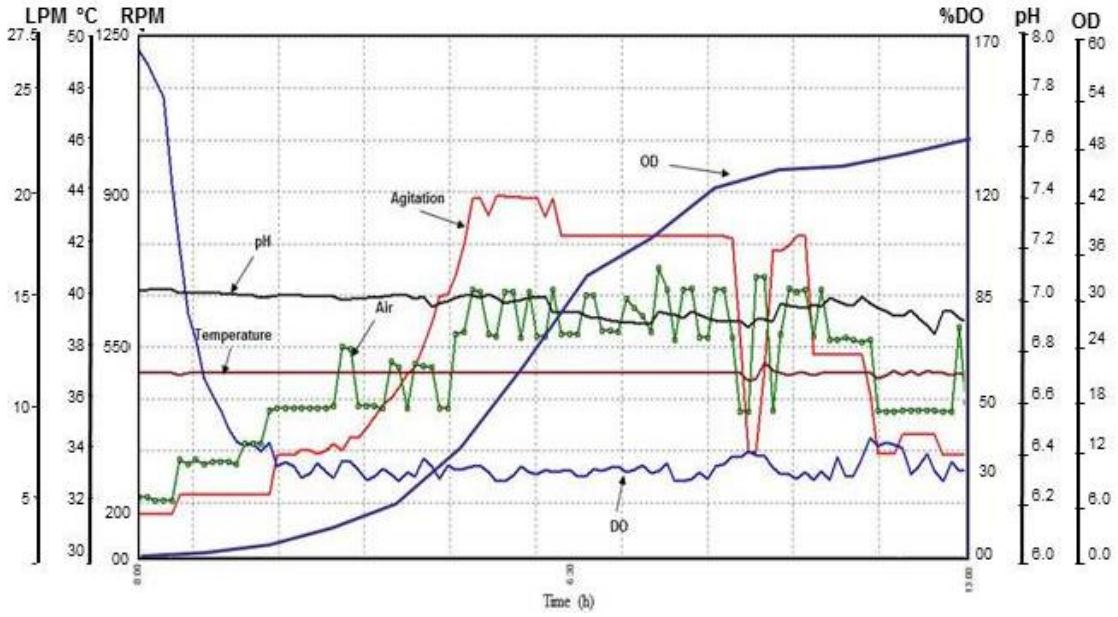

Figure 6.2. Real time profile of fed-batch cultivation for production of rJEV NS1 protein. The culture was induced with 1 mM IPTG (after ~9 h of cultivation) at DCW of 13.41 g/l and cells were grown further for 4 hours to attain DCW of 17.78 g/l.

6.3.3 Purification and Characterization of rJEV NS1 Protein

Expression of heterologous protein in *E. coli* allows its rapid and economical production in large amounts. In an effort to obtain target protein in host *E. coli* strain, IBs formation is still considered as a convenient and effective way in recombinant protein production (Singh and Panda, 2005). After cell disruption and centrifugation analysis of the lysate confirmed the presence of the major proportion of ~44 kDa protein band. IBs were harvested and purified from the induced and lysed cell mass. The IBs were solubilized in buffer containing 8M urea and purified by affinity chromatography under denaturing

conditions. The purified protein was further dialyzed before used for ELISA. The SDS-PAGE profile of eluted protein is shown in figure 6.3a and 6.3b.

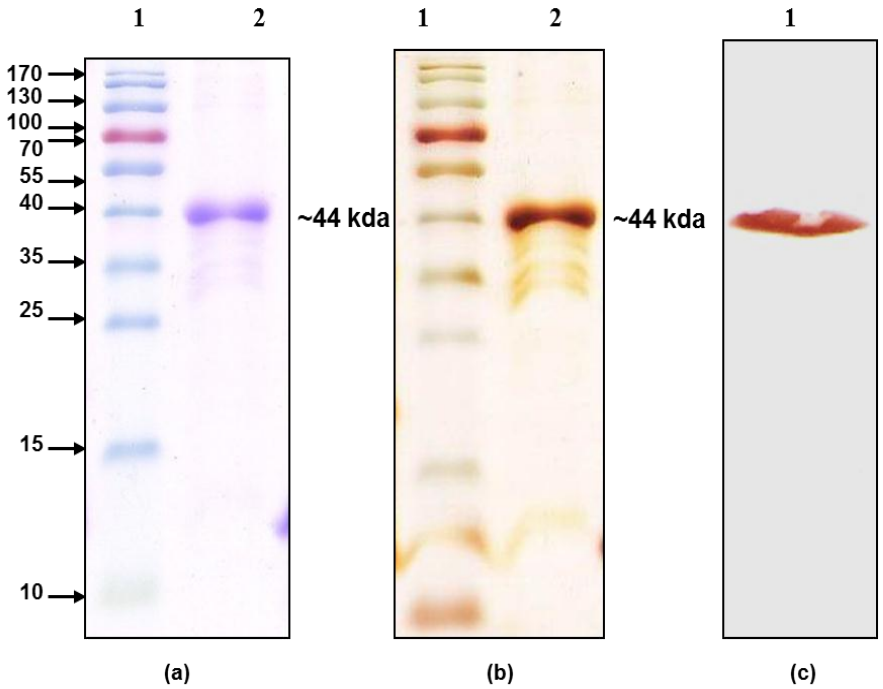

Figure 6.3. (a) Coomassie stained SDS–PAGE. The protein band of ~44.0 kDa confirmed the predicted size of the rJE NS1 protein. The protein profiles of the eluted protein in coomassie stained gel were analyzed densitometrically using Quantity One image quantification software which showed that more than 90% purity has been achieved. Lane 1, Molecular Weight Marker (kDa); lane 2, purified rJEV NS1 protein. (b) Silver stained SDS-PAGE. Lane 1, Molecular Weight Marker (kDa); lane 2, Purified rJEV NS1 protein.(c) Western blot analysis of the purified rJEV protein. The sample showing reaction with protein at desired size (~44 kDa) was considered positive. Lane 1, rJEV NS1 protein with hyper immune serum.

From the protein profiles of the eluted protein in SDS-PAGE (figure 6.3a and 6.3b) and densitometry analysis using Quantity One image quantification software (Bio-Rad, USA), more than 90% purity was found to be achieved. The cell pellet harvested from 50 ml of induced fed-batch culture yielded ~7.0 mg purified rJEV NS1 protein with ~92% purity. This corresponds to a recovery of ~50% as the crude cell lysate was estimated to contain ~13.9 mg of the rJEV NS1 protein and 104 mg total protein, based on densitometric analysis using Quantity One software (Bio-Rad, USA). The solubilized IBs was estimated to contain ~9.0 mg of the rJEV NS1 protein with ~64% recovery and ~75% purity (Table 6.3).

The final product concentration of rJEV NS1 protein following affinity chromatography was significantly higher for fed-batch cultivation as compared to batch cultivation (Table 6.2). Improvement in product yield about more than eight times for fed-batch cultivation as compared to shake flask culture resulted using modified SB medium (Table 6.2). The final rJEV NS1 protein yield after fed-batch cultivation was ~142.16 mg/l (Table 6.2).

Table 6.3. Summary of purification of rJEV NS1 protein from 50 ml bioreactor culture.

Step	Total protein (mg)[a]	Purity (%)[b]	Yield (%)[c]
Total cell lysate	104	0	100
Solubilised IBs	9.0	75	64
Affinity chromatography	7.0	92	49

Total cell lysate represents 50 ml of bioreactor sample of 4 hours post induction.

[a]The protein estimation was carried out using BCA method.

[b]The purity of protein was analysed by coomassie stained SDS-PAGE analysis.

[c]The amount of rJEV NS1 protein in the total cell lysate was ~13.9 mg as determined by densitometric analysis and specified as 100%.

The purified protein was subjected to Western blot assay, to confirm its identity as rJEV NS1 protein. The purified rJEV NS1 protein was tested with mice sera raised against rJEV NS1 protein. This revealed that the rJEV NS1 protein could react with anti-JEV NS1 antibody (Figure 6.3c). It is evident that this protein specifically reacted with anti-JEV antibody and thus suggests that it could be used for the purpose of diagnosis of JEV infection.

4.3.4 Recombinant JEV NS1 Protein as a Diagnostic Reagent

The usefulness of purified rJEV NS1 protein for the detection of anti-JEV IgM antibodies in human sera and CSF samples was carried out by in-house developed indirect dipstick and microwell plate ELISA. A total of 50 clinical samples (30 serum and 20 CSF samples) was included. Optical density (OD_{490}) values of serum and CSF samples determined with in-house microwell plate ELISA are shown in table 6.4. Among 30 serum samples, 16 samples were positive and 14 samples were negative and of 20 CSF samples 12 were positive and 8 samples were negative by in-house dipstick ELISA as well as in-house

microwell plate ELISA. Out of 30 serum samples, 18 samples were positive and 12 samples were negative and of the 20 CSF samples 13 were positive and 7 samples were negative by JEV-CheX IgM capture ELISA.

Table 6.4. Range of OD_{490} values in the in-house indirect microwell plate IgM ELISA for human CSF and serum samples.

In-house ELISA OD range[a]	No. of CSF samples	No. of serum samples
0.000–0.199	08	02
0.200–0.399	05	07
0.400–0.599	04	05
0.600–1.00	03	06
1.00–1.50	-	05
1.50–2.00	-	03
≥2.0	-	02
Total	20	30

[a] ELISA OD_{450} values of ≥ 0.20 and ≥ 0.60 were considered positive at a CSF dilution of 1:10 and serum dilution of 1:100.

Comparison of the both in-house ELISA with JEV Chex IgM capture ELISA revealed comparable sensitivities, specificities and overall agreements (Table 6.5). In addition, 10 healthy serum samples as well as 5 dengue positive serum samples were also included in this study. None of them gave positive result thereby confirming its specificity. The use of rJEV NS1 protein as an antigen in ELISA avoids costly and tedious production of viral antigen as well as the inherent biosafety issues. The diagnosis of JE has advanced considerably in recent years and routine laboratory diagnosis of JE virus infection is primarily carried out by detection of anti-JE virus antibody by serological methods namely MAC-ELISA and indirect IgM ELISA (Plesner and Ronne, 1997; Ravi et al., 2006; Jacobson et al., 2007; Shrivastva et al., 2008; Ravi et al., 2009; Litza et al., 2010; Kumar et al., 2011). Most of these tests are based on the capture principle and or uses native viral antigens (high production cost and produces biohazards). Therefore, there is a genuine need for a promising test system for detection of JEV infection. In the present study, the in-house indirect ELISA test has shown

more than 90% agreement for detection of anti-JEV IgM antibodies in CSF as well as serum samples as compared to commercial assay. The discrepancies in correlation between commercial kit and in-house ELISA (Table 6.5) may be attributed to the use of crude JE virus infected culture fluid as antigen in the commercial kit (Ravi et al., 2006) whereas in-house ELISA uses affinity chromatography purified recombinant JE virus specific nonstructural 1 protein as antigen. The advantages of this in-house ELISA test are that it is cost effective because it uses *E. coli* expressed protein as well as faster than MAC ELISA. Further, the in-house dipstick ELISA is a qualitative test meant for field use. The negative results of rJEV NS1 protein based indirect ELISA with dengue positive human sera as well as negative serum samples established its specificity for JE diagnosis. These findings suggest that the rJEV NS1 protein based indirect ELISA is a sensitive and specific test for early detection of JEV infection.

Table 6.5. Comparative evaluation of in-house ELISA with reference to JEV CheX IgM capture ELISA for detection of anti-JEV IgM antibodies in patient serum and CSF samples.

Type of sample	% Agreement[a]	% Sensitivity[b]	% Specificity[c]
Serum	93%(28/30)	89%(16/18)	100%(12/12)
CSF	95%(19/20)	92%(12/13)	100% (07/07)

[a](Number of samples positive by both method + number of samples negative by both methods) /(total number samples) x100.

[b]True positive/(true positive + false negative) x 100.

[c]True negative /(true negative + false positive) x 100.

Since the recombinant JE virus NS1 protein shown high immunogenicity (Lin et al., 2008), it is useful antigen for immunodiagnosis of JEV infection and also for immunoprophylaxis. For use in serological diagnosis of JEV infection as well as further studies in immunoprophylactic development, large quantity of biologically active protein was required which could be produced using bioreactor. The media optimization, batch and fed-batch cultivation strategies as mentioned in the present study demonstrated its use more appropriately. The results of in-house ELISA using purified rJEV NS1 protein with reference to commercial assay was in good concordance. The findings of present study exhibits that the

rJEV NS1 protein can be an antigen of choice for cost effective serological diagnosis of JEV infection with acceptable specificity, sensitivity and agreement.

6.4 CONCLUSIONS

We have described here a robust and scalable cultivation method to produce recombinant JEV NS1 protein as insoluble form at 10 liter cultivation scale. Usually recombinant *E. coli* is the host of choice for heterologous protein production as it is easier to grow it to higher biomass on inexpensive carbon and nitrogen sources. The composition of media affected the yield of rJEV NS1 protein production. From the economic point of view, it is necessary to increase the biomass as well as amount of protein of interest employing suitable cultivation strategies and efficient purification processes. In this study, the high cell density fed-batch cultivation was adopted to increase the protein yield. A simple, one step purification strategy involving metal affinity chromatography was devised to produce rJEV NS1 protein with high purity. This process resulted in ~142 mg of purified rJEV NS1 protein per liter of culture. The purified rJEV NS1 protein thus produced has a potential application for detection of anti-JEV IgM antibody as well as further studies in vaccine development for JEV infection. This approach of creating recombinant antigens coupled to over-expression in *E. coli* and simple purification offers a promising alternative option to JE diagnosis with the potential to circumvent the drawbacks of the whole virus antigen based assays. The method described here to produce rJEV NS1 protein may also be useful in producing other viral and bacterial proteins in *E. coli* as insoluble form for production at large scale.

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CHAPTER 7

PRODUCTION OF RECOMBINANT DENGUE VIRUS TYPE 3 EDIII PROTEIN BY BATCH AND FED-BATCH FERMENTATION AS WELL AS EVALUATION OF ITS DIAGNOSTIC POTENTIAL

ABSTRACT

Dengue is a public health problem of global significance for which there is neither an effective antiviral therapy nor a preventive vaccine. The envelope protein of dengue virus is the major antigen to elicit neutralizing antibody response and protective immunity in hosts thus making it as a diagnostic and vaccine candidate. Optimization of culture media was carried out for enhanced production of recombinant dengue virus type 3 envelope domain III (rDen 3 EDIII) protein in *E. coli*. Further, batch and fed-batch cultivation process were also developed in optimized medium. After fed-batch cultivation, the recombinant *E. coli* resulted in dry cell weight of about 22.80 g/l of culture. The rDen 3 EDIII protein was purified from inclusion bodies using affinity chromatography. This process produced ~649 mg of purified rDen 3 EDIII protein per liter of culture. The purity of this protein was checked by SDS-PAGE analysis and the reactivity was analyzed by Western blotting. The purified protein was also used to develop an in-house enzyme linked immunosorbent assays and tested with human serum samples. These results show that the purified protein has the potential to be used for the diagnosis of dengue virus infection or for further studies in vaccine development.

7.1 INTRODUCTION

Dengue viruses comprise four antigenically distinct serotypes (1 to 4). Its genome encodes for three structural proteins: the capsid protein, the premembrane protein and the envelope glycoprotein and seven non-structural proteins (Mackenzie et al., 2004). The envelope (E) protein comprises 3 regions: Domain I, II and III. Out of these, domain III (DIII) is an immunoglobulin-like receptor binding domain (Rey et al., 2003; Chin et al., 2007). In addition, it has also been demonstrated to be highly immunogenic and able to elicit the production of neutralizing antibodies against the wild-type virus (Crill and Roehrig, 2001). Envelope DIII (EDIII), which spans amino acids (aa) 300 to 400 of the E protein, is a highly stable, independently folding domain that lies exposed and accessible on the virion surface. Multiple type and subtype specific neutralizing epitopes of the envelope protein have been mapped to EDIII (Kuhn et al., 2002; Gromowski et al., 2007; Guzman et al., 2010).

Since no effective vaccine is available for dengue, early diagnosis plays an important role to control the disease. In a majority of cases the only feasible diagnostic test is based on

the identification of anti-dengue virus IgM and IgG antibodies, which appear after the viremic phase and persist much longer. Recently, several dengue diagnostic tests, in different formats, have become available commercially (Groen et al., 2000; Abhyankar et al., 2006; Blacksell et al., 2006, 2008). Most of them uses infected mouse brain or insect cell extracts. It is associated with inherent biohazard risk, cross-reactivity with related *Flaviviruses*. Replacement of the whole-virus antigens with a mixture of recombinant envelope (E) proteins of the four dengue virus serotypes eliminated the safety risk but not the cross-reactivity problem (Cuzzubbo et al., 2001). However, all these kits are expensive due to the high costs associated with antigen production (AnandaRao et al., 2006), making them unaffordable for use in the economically weaker countries where dengue occur most frequently. In an attempt to eliminate this cross-reactivity, we have focused on a discrete domain of the E protein, known as domain III (EDIII). Earlier studies suggested that the envelope domain III protein of dengue virus can be used for detection of anti-dengue virus antibodies (Simmons et al., 1998; Jaiswal et al., 2004; Hapugoda et al., 2007; Pattnaik et al., 2007) and also for vaccine studies (Jaiswal et al., 2004; Chen et al., 2007; Pattnaik et al., 2007; Babu et al., 2008; Sim et al., 2008). For this reason, the EDIII protein is an important immunogen for the development of a prospective protein subunit vaccine and also a prospective diagnostic reagent for the improved clinical diagnosis of dengue infections.

A high cell density cultivation technique is important to maximize volumetric productivity. *Escherichia coli* is the most commonly used host for heterologous protein production because it is a well-characterized organism in the genetics, physiology and cultivation condition (Lee, 1996; Panda, 2003; Bhatnagar et al., 2008). Recombinant *E. coli* can be grown to high densities in common media such as Luria bertani broth, SOB medium and Super broth medium (Madurawe et al., 2000; Manderson et al., 2006). Growing *E. coli* to high density using fed-batch cultivation process is currently the method of choice for the production of recombinant proteins, mainly because of the high volumetric productivity associated with this method (Babu et al., 2000; Khalilzadeh et al., 2004, 2008). However, high cell density cultures also have a few drawbacks such as substrate inhibition, the formation of growth inhibitory by-products. The composition of the growth media is crucial for enhancing product formation as well as reduction of inhibitory compound formation (Manderson et al., 2006; Tripathi et al., 2009).

There is currently a need for developing cultivation process for enhanced production of recombinant dengue virus type 3 envelope domain III (rDen 3 EDIII) protein and development of cost-effective, safe and simple diagnostics with sensitivity, specificity and applicability in laboratory as well as field conditions. In order to produce recombinant proteins in *E. coli* with high yield, over-expression of the recombinant protein in a cultivation process and a purification procedure allowing efficient recovery of the protein from the resultant biomass are necessary. Thus, we have focused on the production of rDen 3 EDIII protein from recombinant *E. coli* which over-expressed this protein in the form of insoluble inclusion bodies. The media optimization, batch and fed-batch cultivation were performed to maximize overall productivity of rDen 3 EDIII protein. Further, evaluation of this protein was carried out for its potential as a diagnostic tool using dipstick ELISA for the detection of anti-dengue virus IgM and IgG antibodies using patient serum samples.

7.2 MATERIALS AND METHODS

The details about materials used for production of rDen 3 EDIII protein are desribed in the material and methods section (Chapter 3). The methods for expression, media optimization, small scale batch fermentation, fed-batch fermentation, affinity chromatography, SDS-PAGE, Western blot and ELISA are described in the materials and methods section (Chapter 3). A typical flow diagram for production and purification of rDen 3 EDIII protein used as a diagnostic reagent is similar to that shown in figure 4.1.

7.3 RESULTS AND DISCUSSION

7.3.1 Expression of Recombinant Dengue Virus type 3 EDIII Protein

The present study was envisaged to produce large amount of rDen 3 EDIII protein and its evaluation as a diagnostic reagent for Dengue. To produce this protein in *E. coli*, a synthetic gene encoding Den 3 EDIII protein was previously cloned in pET30a+ bacterial expression vector. This construct is predicted to encode a recombinant protein with an approximate molecular weight of ~12 kDa. To express recombinant dengue virus type 3 envelope domain III protein in *E. coli*, shake flask culture was carried out in LB medium. A typical induction experiment comparing the profiles of un-induced and IPTG-induced *E. coli* cultures for dengue virus serotype 1-4 is shown in Figure 7.1.a. It is evident that IPTG induction results in the expression of a ~12 kDa Den 3 EDIII protein. Growth culture induced

with different concentration of inducer (0.5, 1.0 and 1.5 mM IPTG) at 4 h post induction time has shown the maximum protein expression with 1 mM IPTG (Figure 7.1.b). Therefore, in all subsequent experiments induction was carried out with 1 mM IPTG for 4 h.

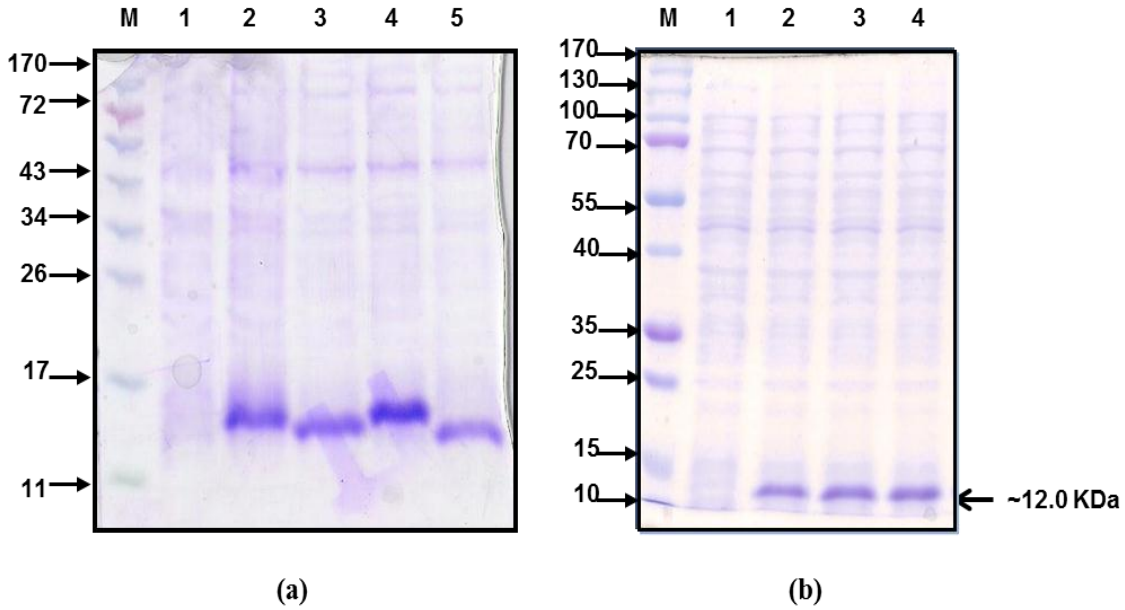

Figure 7.1. SDS-PAGE analysis of expression of rDen 1-4 EDIII protein. (a). Lane M : MW marker (kDa) ; lane 1: Un-induced culture lysate ; lane 2-5: Induced culture lysate expressing rDen 1-4 EDIII protein respectively (~12.0 kDa). (b) lane 1: Un-induced culture lysate ; lane 2-4: 0.5, 1.0 and 1.5 mM IPTG induced culture lysate expressing rDen 3 EDIII protein (~12.0 kDa).

7.3.2 Effect of Media on Production of rDen 3 EDIII Protein

Composition of media seem to be important to ensure proper cell growth and for rDen 3 EDIII protein production. The present study therefore had an objective to produce rDen 3 EDIII protein with a high yield and evaluate its use in diagnosis of the disease. In the shake flask culture, final cell density and cell concentration was found to depend upon the media used. The growth profile of *E. coli* cells expressing this protein is shown in figure 7.2. Super broth achieved final dry cell weight of about 2.42 g/l. SOB medium resulted in final dry cell dry weight about 1.41 g/l. However, LB medium produced 1.34 g/l dry cell weight. High-cell densities attained with SB can be explained, as these media are rich in yeast extract and phosphate salts compared to the other media used. Production characteristics of rDen 3 EDIII

protein expressed in *E. coli* in terms of OD, dry cell weight and rDen 3 EDIII protein yield at shake flask culture using different media are summarized in table 7.1.

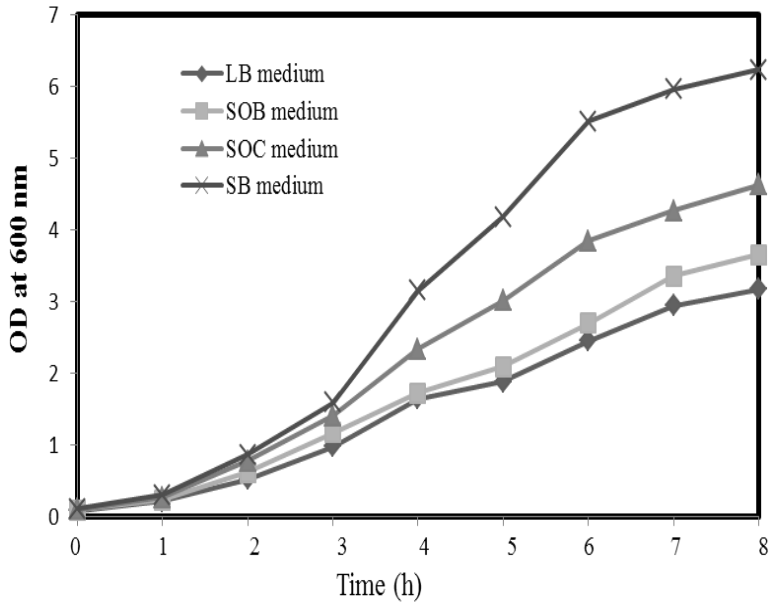

Figure 7.2. Growth profile of recombinant E. coli in shake flask culture with four different culture media.

Table 7.1.Production characteristics of rDen 3 EDIII protein expressed in *E. coli* using different media in shake flask culture.

Media	OD_{600}	DCW (g/l)	rDen 3 EDIII Protein (mg/l)
LB Broth	3.18	1.34	39.25
SOB	3.66	1.41	42.16
Super Broth	6.24	2.42	54.64

7.3.3 Production of rDen 3 EDIII Protein in *E. coli*

The optimization of media for rDen 3 EDIII protein production was also performed in the bioreactor using batch mode under controlled cultivation parameters. In batch cultivations with LB, SOB and SB medium, the dry cell weight at induction was 0.96, 1.32 and 2.30 g/l respectively. The final dry cell weight at harvest in all three media is shown in table 7.2. We found that the SB medium again resulted in more OD, dry cell weight and level of rDen 3 EDIII protein expression in comparison with LB and SOB media (Table 7.2). This may be

due to the presence of yeast extract and or glycerol in comparison with other media. For further maximizing the volumetric production, *E. coli* cells expressing rDen 3 EDIII protein were grown in a fed-batch cultivation process using optimized medium. The cultures at cell OD of 42.79 (15.10 g/l dry cell weight) were induced with 1 mM IPTG and grown for another 4 hours. The growth profile (OD vs. Time) during course of fed-batch cultivation is shown in figure 7.3. Once feeding is initiated and *E. coli* enters into log phase (Figure 7.3), the feed is consumed more or less in an exponential manner. Therefore, the feeding rate has to be controlled so that it doesn't exceed the nutrient demand or feed consumption rate. It is done by maintaining the pH and DO around their set values. Rise in pH and DO values

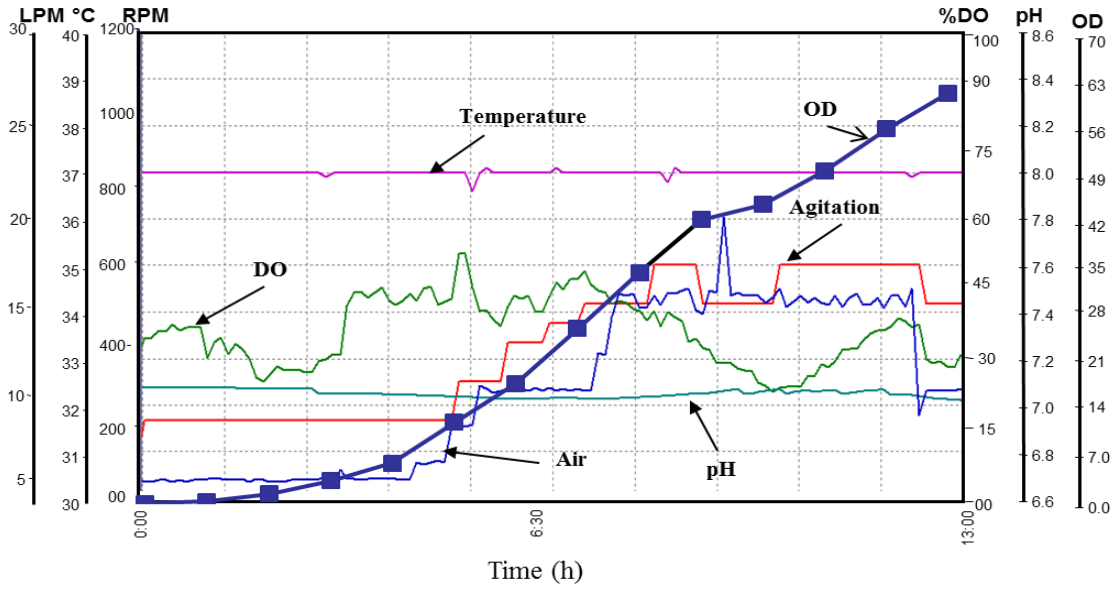

Figure 7.3. Real time profile of fed-batch cultivation for production of rDen 3 EDIII protein. The culture was induced with 1 mM IPTG (after ~9 h of cultivation) at OD of 42.79 and cells were grown further for 4 hours. The culture OD at harvest (after ~13 h of cultivation) was 62.13.

indicate that the carbon source or one of the substrates is limiting and hence feed is required. Yeast extract present in growth media help in reducing secretion of acetic acid as well as utilisation of acetic acid during carbon limitation at the time of growth of *E. coli*. Yeast extract have also been reported to enhance the specific cellular yield of the expressed protein particularly during high cell density cultivation where the demand of nitrogenous source becomes very high following induction (Panda, 2003; Zhang et al., 2010). Glycerol is used as a carbon source and high cell densities with less frothing (anti foaming effect) may be

achieved relatively easily using glycerol. The final dry cell weight of about 22.80 g/l was attained at ~13 h of cultivation (Table 7.2). Real time profile of fed-batch cultivation for production of rDen 3 EDIII protein is shown in figure 7.3. Comparative evaluation of OD, dry cell weight and rDen 3 EDIII protein in batch and fed-batch cultivation using different media is shown in table 7.2. Reproducibility of all batch and fed-batch cultivations was confirmed with additional experiments conducted under the above specified optimal conditions and the final yield was within less than 10% of the result shown in table 7.2. The final dry cell weight after fed-batch cultivation was about seventeen times more in comparison with shake flask culture using commonly used LB medium and about nine times more with SB medium.

Table 7.2. Production characteristics of rDen 3 EDIII protein expressed in *E. coli* using different media in bioreactor.

Media	OD_{600}	DCW (g/l)	rDen 3 EDIII Protein (mg/l)
LB Broth[b]	5.35	2.10	66.13
SOB[b]	8.28	2.96	97.60
Super Broth[b]	13.14	4.50	176.76
Super Broth[c]	61.78	22.80	649.24

[b]Batch cultivation; [c]Fed-batch cultivation

7.3.4 Purification and characterization of rDen 3 EDIII protein

The present study demonstrated the feasibility of obtaining pure rDen 3 EDIII protein utilizing a single chromatographic step using affinity chromatography. The inclusion bodies were solubilized in buffer containing 8 M urea and purified by affinity chromatography under denaturing conditions. The SDS-PAGE profile of rDen 3 EDIII protein is shown in figure 7.4a. From a comparison of the protein profiles of the eluted protein, solubilized IBs and the crude cell lysate in silver stained SDS-PAGE (Figure 7.4a) densitometrically, it is clearly evident that more than 95% purity has been achieved. The cell pellet harvested from 50 ml of induced fed-batch culture yielded ~32 mg purified recombinant dengue virus type 3 envelope domain III protein. This corresponds to a recovery of ~52% as the crude cell lysate was estimated to contain ~61.5 mg of the recombinant protein (Table 7.3), based on densitometric analysis.

Table 7.3. Summary of purification of rDen 3 EDIII protein from bioreactor culture.

Step	Total protein (mg)[a]	Purity (%)[b]	Yield (%)[c]
Total cell lysate	510	0	100
Solubilised IBs	40	80	65
Affinity chromatography	32	95	52

Total cell lysate represents 50 ml of bioreactor sample of 4 hours post induction.

[a]The protein estimation was carried out using BCA method.

[b]The purity of protein was analysed by silver stained SDS-PAGE analysis.

[c]The amount of rDen 3 EDIII protein in the total cell lysate was ~61.5 mg as determined by densitometric analysis and specified as 100%.

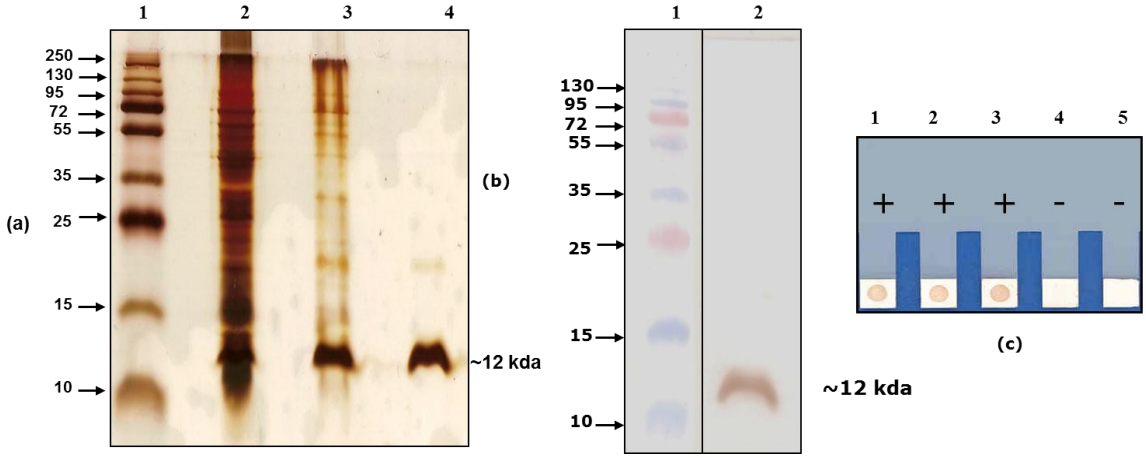

Figure 7.4. (a) Silver stained SDS-PAGE. The protein band at approximate molecular weight of ~12.0 kDa confirmed the predicted size of the rDen 3 EDIII protein. The protein profiles of the eluted protein, solubilized IBs and the crude cell lysate in silver stained gel were analyzed densitometrically which showed that more than 95% purity has been achieved. Lane 1, Molecular Weight Marker (kDa); lane 2, Total cell lysate; lane 3, Solubilized IBs; lane 4, Purified rDen 3 EDIII protein. (b) Western blot analysis showing the reactivity of serum sample with purified rDen 3 EDIII protein. Lane 1, Molecular weight marker; lane 2, rDen 3 EDIII protein with anti-dengue IgG positive serum samples. (c) Results of Dipstick ELISA with rDen 3 EDIII protein. Lane 1, Positive control; lane 2, IgM positive; lane 3, IgG positive; lane 4, Negative control; lane 5, Both (IgM and IgG) negative serum sample.

The final product concentration of rDen 3 EDIII protein following affinity chromatography was significantly higher for fed-batch cultivation as compared to batch

cultivation (Table 7.2). Improvement in product yield about more than eleven times for fed-batch cultivation as compared to shake flask culture resulted using SB medium (Table 7.1 and 7.2). The final rDen 3 EDIII protein yield after fed-batch cultivation was ~649.24 mg/l (Table 7.2).

The purified protein was subjected to Western blot assay, to confirm its identity as rDen 3 EDIII protein. The affinity purified rDen 3 EDIII protein was tested with dengue positive serum sample. The results revealed that the dengue positive human serum sample reacted with rDen 3 EDIII protein (Figure 7.4b). It is evident that the rDen 3 EDIII protein reacted with anti-dengue virus antibody and thus suggests that this protein could be used for the purpose of diagnosis of dengue virus infection.

7.3.5 Evaluation of rDen 3 EDIII protein by in-house ELISA

Evaluation of the utility of this protein as a diagnostic tool for the detection of both anti-dengue virus IgM and IgG antibodies using patient serum samples was carried out by in-house developed dipstick as well as microwell plate ELISA. In the present study, a panel of 90 human serum samples from patients with suspected dengue infections was included. OD_{450} values for all serum samples determined by Pan-Bio IgM and IgG capture ELISA are shown in table 7.4.

Table 7.4. Range of OD_{450} values in the IgM and IgG capture ELISA for human serum, used to evaluate in-house ELISA.

Capture ELISA OD range[a]	No. of serum samples[b]	No. of serum samples[c]
0.000–0.099	26	14
0.100–0.199	12	21
0.200–0.399	10	12
0.400–0.599	22	17
0.600–0.999	14	16
≥1.00	6	10
Total	**90**	**90**

[a] ELISA OD_{450} values of ≥ 0.20 were considered positive at a serum dilution of 1:100 for IgM antibodies and of 1:1000 for IgG antibodies.
[b] No of serum samples in IgM capture ELISA.
[c] No of serum samples in IgG capture ELISA.

OD_{490} values for all serum samples determined by in-house microwell plate ELISA are shown in table 7.5. Table 7.6 shows the comparative results of detection of anti-dengue virus IgM antibodies. Among 90 samples, 49 samples were positive and 41 samples were negative by in-house ELISA using rDen 3 EDIII protein. Out of 90 samples 52 samples were positive and 38 samples were negative by Pan-Bio IgM capture assay and 50 samples were positive and 40 samples were negative by Pan-Bio rapid IC test.

Table 7.5. Range of OD_{490} values in the in-house indirect IgM and IgG microwell plate ELISA for human serum samples.

ELISA OD range[a]	No. of serum samples[b]	No. of serum samples[c]
0.000–0.149	29	25
0.150–0.249	12	11
0.250–0.499	7	3
0.500–0.799	23	13
0.800–0.999	10	24
≥1.00	9	14
Total	**90**	**90**

[a] ELISA OD_{490} values of ≥ 0.25 were considered positive at a serum dilution of 1:100 for IgM antibodies and of 1:1000 for IgG antibodies.

[b] No of serum samples in IgM ELISA; [c] No of serum samples in IgG ELISA.

Table 7.7 also shows the comparative results of detection of anti-dengue IgG antibodies. Among 90 samples, 54 samples were positive and 36 samples were negative by in-house ELISA using rDen 3 EDIII protein. Out of 90 samples 55 samples were positive and 35 samples were negative by Pan-Bio IgG capture assay and 52 samples were positive and 38 samples were negative by Pan-Bio rapid IC test.

Table 7.6. Comparative evaluation of in-house ELISA with reference to rapid IC test and IgM capture ELISA for detection of dengue IgM antibodies.

Test	n	% Agreement[a]	% Sensitivity[b]	% Specificity[c]
Dipstick ELISA/ IC Test	90	98% (89/90)	98% (49/50)	100% (40/40)
Dipstick ELISA/ Capture ELISA	90	96% (87/90)	94% (49/52)	100% (38/38)

n, total number of sera tested.

[a] (Number of samples positive by both method + number of samples negative by both

methods) /(total number samples) x100.

[b] True positive/(true positive + false negative) x 100.

[c] True negative /(true negative + false positive) x 100.

Comparison of the in-house dipstick and microwell plate ELISA using rDen 3 EDIII protein with Pan-Bio rapid IC test, Pan-Bio IgM and IgG capture ELISA for detection of IgM and IgG antibodies to dengue revealed comparable sensitivities, specificities and overall agreements (Table 7.6 and 7.7). The cut-off value (OD_{490}) for in-house microwell plate ELISA was 0.25 for anti-dengue IgM and IgG antibodies detection. The cut-off value for Pan-Bio capture ELISA was determined by taking average of OD_{450} values of cut-off calibrator in triplicate as per manufacturer's protocol.

Table 7.7. Comparative evaluation of in-house ELISA with reference to rapid IC test and IgG capture ELISA for detection of dengue IgG antibodies.

Test	n	% Agreement[a]	% Sensitivity[b]	% Specificity[c]
Dipstick ELISA/ IC Test	90	97% (88/90)	100% (52/52)	94% (36/38)
Dipstick ELISA/ Capture ELISA	90	98% (89/90)	98% (54/55)	100% (35/35)

The routine laboratory diagnosis of dengue virus infection is primarily carried out by detection of anti-dengue antibody by serological methods. A number of serological tests namely μ-capture ELISA, dot ELISA and imunochromatography based assays were evaluated and used successfully by various investigators (Porter et al., 1999; Wu et al., 2000; Abhyankar et al., 2006; Blacksell et al., 2008, 2008). IgM antibody detection is a crucial and reliable assay for samples collected from 5^{th} day of onset of symptoms (Lindegren et al., 2005). The extensive evaluation revealed disparities among various commercial kits with respect to their sensitivity and specificity (Lindegren et al., 2005). However, the Pan-Bio IgM capture ELISA is the most reliable and widely used owing to its high sensitivity and specificity. Though a large number of rapid diagnostic tests (RDTs) are available in the market, however, their diagnostic accuracy falls well below the manufacturer's claim (Blacksell et al., 2006). Therefore, there was a genuine need for a promising test system for detection of dengue infection. In the present study an in-house ELISA was designed for use in the laboratory as well as in the field. The rDen 3 EDIII protein obviates expensive and time consuming cultivation of virus (for antigen preparation) and the associated biohazard

risk. The reaction pattern of in-house developed dipstick ELISA using rDen 3 EDIII protein with positive and negative serum samples is shown in figure 7.5c. In comparison with other assays used in this study, the in-house ELISA showed more than 95% accordance. The IC test using a mixture of recombinant envelope protein of dengue virus 1 to 4 has been developed and reported to be sensitive but less specific (AnandaRao et al., 2006). The rDen 3 EDIII protein could react with sera against all the four serotypes because there lies a common antigenic epitope for the four serotypes (Innis et al., 1989; Gubler, 1998; AnandaRao et al., 2006; Tan and Ng, 2010). Dengue 2 NS1-P1 peptide, corresponding to amino acid (aa) residues 1 to 15 of dengue-2 NS1, a linear, immunodominant IgM-specific epitope which was reactive towards patient sera from all four dengue serotypes has been reported (Huang et al., 1999). The amino acid sequences of domain III protein of all four dengue virus serotypes were aligned and analyzed which revealed that the amino acid similarity for DIII protein of dengue virus type 3 with that of dengue virus type 1, type 2 and 4 is 87.9%, 79.8% and 69.7% respectively (Tan and Ng, 2010). Evaluation of rDen 3 EDIII protein with hyper immune sera against all the serotypes of dengue viruses showed positive reaction and thereby confirming its sensitivity for the diagnosis of dengue infection. Further this protein did not react with a panel of defined sera samples from 20 apparently healthy individuals. The amino acid sequence alignment of the domain III protein across the *Flaviviridae* family was performed which revealed that there are significant differences in the homology of the domain III amino acid sequences across *flaviviruses* (Chu et al., 2004). Earlier studies suggested the utility of domain III protein from dengue virus type 1-4 or JE virus as antigens for specific serological diagnosis of infections with those flaviviruses (Fonseca et al., 1991; Simmons et al., 1998). Further, West Nile virus recombinant domain III was found to be sensitive and very specific for West Nile virus infection and could also differentiate between closely related mosquito-borne flaviviruses. The recombinant domain III protein of West Nile virus was also found to be superior to whole virus antigens in discriminating specific antibody responses to West Nile virus (Beasly et al., 2004).

The domain III of the envelope protein is particularly an important antigen for vaccine development as well as its use as a reagent for diagnostic purposes**,** as it contains multiple serotype specific conformation dependent neutralizing epitopes and host cell receptor recognition site. The antibodies raised against domain III may block the entry of

virus in to the cell. Choosing only domain III as a vaccine candidate instead of whole envelope protein has the advantage of reducing the menace of ADE because of the absence of other epitopes which elicit non-neutralizing, cross-reactive antibodies (Guzman et al., 2010; Tan and Ng, 2010). Increase in the production of rDen 3 EDIII protein for application in diagnostic use as well as for further vaccine studies was necessary. Optimization of cultivation medium and cultivation conditions as described in this report, made its application more feasible. Further**,** the use of rDen 3 EDIII protein as an antigen in ELISA resulted in excellent agreement with the findings of commercial rapid IC test and capture ELISA. These results show that the product has a promising potential for its use in diagnosis of dengue with comparable sensitivity and specificity.

7.4 CONCLUSIONS

An efficient cultivation process has been demonstrated to produce large amount of biologically active rDen 3 EDIII protein. From the economic point of view, it is necessary to increase the yield of protein of interest. Recombinant *E. coli* is the most widely used organism for recombinant protein production because of its rapid growth to high-cell densities on inexpensive substrates and its well-characterized genetics and proteomics. Production of recombinant dengue virus type 3 envelope domain III protein was influenced by the media composition. For cost-effective reasons, it is important to maximize protein production by development of efficient cultivation process and appropriate purification methods. In this study, the fed-batch process yielded ~649 mg of purified rDen 3EDIII protein per liter of culture. These result exhibit the ability to produce high yield of dengue virus protein. The biologically functional rDen 3 EDIII protein thus produced has a potential application for detection of anti-dengue IgM and IgG antibodies as well as further studies in vaccine development for dengue virus infection. By using this strategy, other recombinant proteins can be produced at large scale with high yield and purity.

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CHAPTER 8

DEVELOPMENT OF A PILOT SCALE PRODUCTION PROCESS FOR RECOMBINANT DENGUE VIRUS TYPE 3 EDIII PROTEIN AND CHARACTERIZATION FOR ITS VACCINE POTENTIAL

ABSTRACT

Dengue virus infection poses a serious global public health threat for which there is currently no therapy or a licensed vaccine. Envelope domain III protein of dengue virus is immunogenic and induced protective antibodies. In order to take this vaccine candidate for further studies, we developed a scalable process to produce and purify biologically active recombinant dengue virus type 3 envelope domain III (rDen 3 EDIII) protein. This protein was produced in *E. coli* by batch process using a 100 liter fermentor. The cells were harvested using tangential flow microfiltration followed by centrifugation. The cells were disrupted by bead milling and inclusion bodies were solubilized in urea. The rDen 3 EDIII protein was refolded and subsequently purified using immobilized metal affinity chromatography. The purity of protein was further improved by salt and pH based ion exchange chromatography. The purified protein was characterized for its purity by SDS-PAGE and for reactivity by western blot analysis. The final yield of pilot scale produced protein after on-column refolding and affinity purification resulted in approximately 29 mg per g dry cell weight. Biological function of the refolded and purified protein was confirmed by their ability to generate rDen 3 EDIII specific antibodies in mice that could neutralize the virus. The results presented here exhibit the ability to generate multi-gram quantities of rDen 3 EDIII protein from *E. coli* that may be used for the development of dengue vaccines.

8.1 INTRODUCTION

Dengue is the most important mosquito-borne *flavivirus* disease. People living in the tropical and subtropical areas are at risk of dengue virus infection, and more than 50 million dengue infected cases occur worldwide each year (Halstead et al., 2007; Guzman et al., 2010). Vaccine inoculation is a cost-effective way of combating the threat of infectious diseases. In the past six decades, tremendous effort has been made to develop a dengue vaccine (Whitehead et al., 2007; Coller and Clements, 2011; Schmitz et al., 2011). However, despite these efforts, no licensed dengue vaccines are currently available. Many advanced biological technologies have been applied to dengue vaccine development, and numbers of vaccine approaches are currently in pre-clinical or clinical development. These approaches include chimerization with other flaviviruses or the deletion of portions of the genomes to obtain live attenuated dengue vaccines, viral vector vaccines, DNA vaccines, and

recombinant subunit vaccines (Whitehead et al., 2007; Coller and Clements, 2011; Schmitz et al., 2011; Chiang et al., 2012). All of the approaches are associated with different advantages and disadvantages. Among these approaches, the recombinant subunit vaccine provides the greatest degree of safety. The envelope protein comprises 3 regions: Domain I, Domain II and Domain III. Experimental evidences have shown that the EDIII protein is a receptor binding domain (Guzman et al., 2010; Block et al., 2010; Coller et al., 2011; Simmons et al., 2012). In addition, it has also been demonstrated to be highly immunogenic and able to elicit the production of neutralizing antibodies against the wild-type virus (Tan et al., 2010; Chiang et al., 2012). One major challenge to dengue virus vaccine development is the potential development of antibody-dependent enhancement (ADE) of virus replication, which is believed to cause DHF and DSS (Simmons et al., 2012). In addition, immunization against one dengue serotype induces life-long immunity against the homologous serotype and short-lived immunity against the other serotypes. Put together, it is widely believed that for a dengue virus vaccine to be effective, it must comprise neutralizing epitopes from all four serotypes (tetravalent) (Tan and Ng, 2010). For this reason, the DIII protein is believed to be a potential candidate as a protein subunit vaccine. Currently, there is a need for the production of cost effective and safe recombinant EDIII protein for the development of protein subunit vaccines. For these purposes, the recombinant proteins produced must maintain their biological activity (i.e., generate neutralizing antibodies against wild-type virus or able to bind to anti-dengue antibodies found in patient serum).

The EDIII proteins may be expressed using various hosts, such as bacteria, yeast and even in the leaves of tobacco plants (Etemad et al., 2008; Guzman et al., 2010). *E. coli* is by far the most commonly used host for the production of EDIII proteins. Small scale expression is widely used for optimizing conditions for a large-scale production of recombinant proteins. The up-scaling process of rEDIII protein production may be performed by replacing commonly used shake flasks (Batra et al., 2010; Tan et al., 2011; Chiang et al., 2012) to fed-batch or batch fermentation in a fermentor. To facilitate the purification of EDIII proteins, the proteins are commonly produced as fusion proteins that comprise of the DIII protein fused with an affinity tag, such as the hexahistidine tag. After the preliminary purification using affinity chromatography, the purity level of these EDIII proteins can be further enhanced by ion exchange or size exclusion chromatography (Tan and Ng, 2010). We

described small scale production of envelope domain III protein of dengue virus type 3 from *E. coli* for diagnostic use. Using this work as a starting point, we detail here a scalable process for the production, refolding and purification of rDen 3 EDIII protein for vaccine studies. Further, we present data demonstrating that the antibodies raised in mice against refolded and purified protein neutralize the dengue virus type 3. The rDen 3 EDIII protein was highly pure and found to be biologically active. This material would be an excellent starting material for the development of new dengue vaccines.

8.2 MATERIALS AND METHODS

The details about materials and methods used for pilot scale fermentation, on-column refolding, ion exchange chromatography, immunogenicity studies and plaque reduction neutralization test are described in the material and methods section (Chapter 3). Figure 5.1 provides an overall flow diagram for the production of rDen 3 EDIII antigen similar to process of rJEV EDIII protein.

8.3 RESULTS AND DISCUSSION

Developing a dengue vaccine has been a challenging task because of the need to provide solid and long-lasting immunity to four serotypes without causing the potentially fatal ADE leading to DHF/DSS. Since conventional approaches based on live attenuated dengue viruses have not resulted in a licensed vaccine, increasing attention is being focused on recombinant strategies (Coller and Clements, 2011; Chiang et al., 2012). These approaches, which are geared towards developing monovalent vaccines targeting single dengue serotype, envisage the creation of a tetravalent vaccine by physically mixing the four monovalent components into a single formulation. To conduct vaccine studies with rDen 3 EDIII antigen, sufficient quantities of purified protein material was required. We previously transformed *E. coli* BLR (DE3) cells using the plasmid (pET 30a+rDen 3 EDIII) construct used to produce small quantities of the protein at lab-scale and using this clone developed a pilot scale process to produce biologically active rDen 3 EDIII protein in good quantities with sufficient purity for use in vaccine studies.

8.3.1 Pilot Scale Production of Recombinant Dengue Virus type 3 EDIII Protein

Although lab scale production of rDen 3 EDIII protein yielded significant protein levels, the potential requirement for this protein in larger quantity led to its production in

pilot scale fermentor. Since the primary goal of large scale fermentation research is cost-effective production of recombinant products, it is important to develop a pilot scale fermentation method that allows the maximization of the yields of the desired product (Ravi et al., 2008; Bell et al., 2009). Production of important foreign proteins in *E. coli* expression system has been highly successful in recent past as this system offers several advantages over the other expression systems, including its fast growth rate, utilization of cheap growth media, high level of expression of foreign proteins (Sahdev et al., 2008; Huang et al., 2012).

The pilot scale batch fermentation process for production of rDen 3 EDIII protein was carried out using 100 liter working volume fermentor with 80 liter modified SB medium. This fermentation process continued for nine and half hour which comprised the pre-induction (five and half hour) and post induction (four hour) phase. At the beginning of the fermentation DO was above 30% and decreased during course of process. The DCW and OD_{600} increased continuously and reached a final value of 6.82 g/l (Table 8.1) and 21.15 respectively. The DCW at induction was 3.12 g/l.

Table 8.1. Production characteristics of rDen 3 EDIII protein for vaccine studies.

Media	Culture Condition	Dry cell weight (g/l)	Protein[c] (mg/l)
Modified SB medium	Shake flask	2.48	63.64
Modified SB medium	Batch fermentation[a]	6.74	185.24
Modified SB medium	Batch fermentation[b]	6.82	201.36

[a]At 5.0 liter scale; [b]At 100 liter scale; [c]After on-column refolding with IMAC

The real time profile of pilot scale batch fermentation is shown in figure 8.1. Before pilot scale fermentation, laboratory scale (5.0 liter working volume) batch process was also carried out using same medium to ensure success of scale up process.

The DCW at induction and harvest after batch fermentation using 5 liter fermentor was 3.08 g/l and 6.74 g/l (Table 8.1) respectively. The real time profile of laboratory scale batch fermentation is shown in figure 8.2.

The critical factor for the development of any vaccine is the ability to produce the vaccine with a process that is amenable to scale-up, is reproducible and produces a reasonable number of doses of the vaccine. Process development is the technological

foundation that underlies the manufacture of new vaccines and is central to successful commercialization (Buckland, 2005). Based on the earlier studies on rDen 3 EDIII protein,

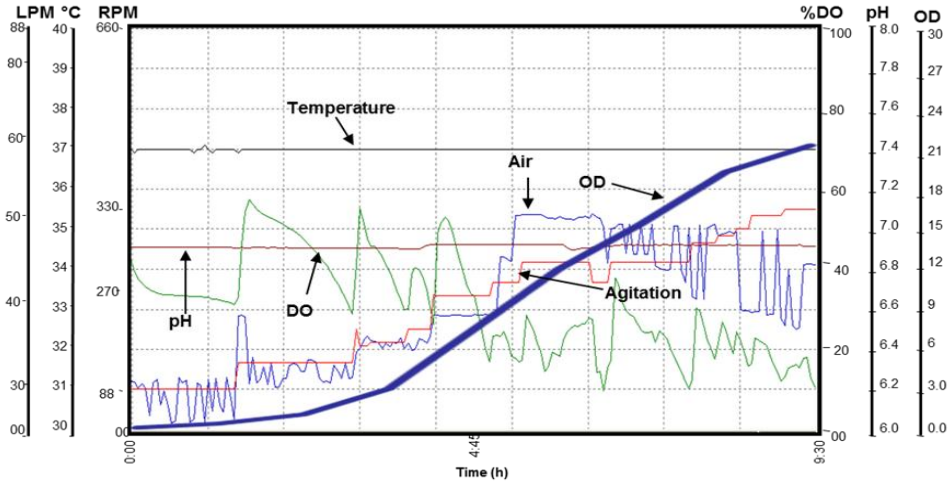

Figure 8.1. Real-time profile of pilot scale batch fermentation for the expression of rDen 3 EDIII protein. Culture was grown in batch mode in modified SB media and induced with 1 mM IPTG at DCW of 3.12 g/l. Figure shows time profile for agitation (rpm), DO concentration (%), air flow rate (liter per min), temperature (^oC), pH and OD_{600}.

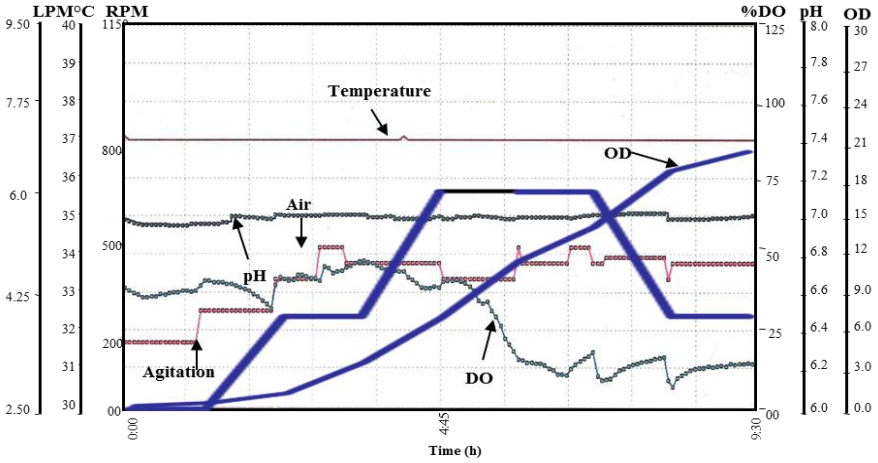

Figure 8.2. Real-time profile of laboratory scale batch fermentation for the expression of rDen 3 EDIII protein. Figure shows time profile for agitation (rpm), DO concentration (%), air flow rate (liter per min), temperature (^oC), pH and OD_{600}.

we envisaged to produce this vaccine candidate on large scale using pilot scale fermentor. Pilot scale fermentation was carried out using modified SB medium. Modified SB medium comprised higher concentration of glycerol compared to other media. Glycerol is one of the

commonly used carbon source. By using glycerol, high dry cell weight and cell densities may be achieved with less frothing (Khamduang et al., 2009). To the best of our knowledge, this is the first report of producing rDen 3 EDIII protein in a 100 liter fermentor. The successful scalable production of this protein in fermentor with a high yield was initiated to foster further applications and research with dengue vaccines.

8.3.2 Refolding and Purification of rDen 3 EDIII Protein

We developed purification process for this protein to obtain a high quality protein suitable for dengue vaccine studies. Purification of rDen 3 EDIII protein was performed from the cell pellets obtained from pilot scale fermentations. The immobilized metal affinity chromatography with simultaneous refolding and cation exchange chromatography based on salt followed by pH was used to obtain highly pure rDen 3 EDIII protein. The real time profile (Time vs OD_{280}) of on-column refolding with affinity purification is shown in figure 8.3. The final product concentration of rDen 3 EDIII protein following IMAC was significantly higher for pilot scale batch fermentation as compared to shake flask culture. Improvement in DCW (2.5-fold higher) and product yield (3-fold higher) resulted for batch fermentation as compared to shake flask culture (Table 8.1).

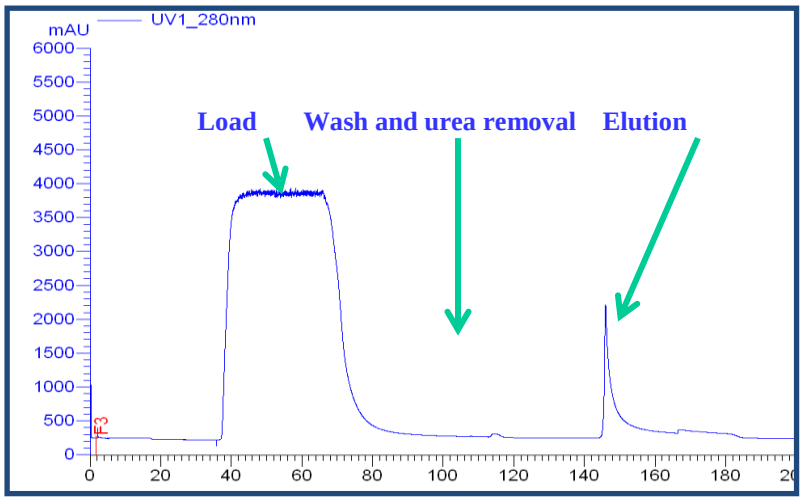

Figure 8.3. Chromatogram showing simultaneous refolding and affinity chromatography for rDen 3 EDIII protein.

However, there was a drop in the yield of rDen 3 EDIII protein following cation exchange chromatography using salt and pH with diafiltration. This observation also indicates that the higher yield of rDen 3 EDIII protein at affinity purification with

simultaneous refolding stage may not necessarily lead to higher yield of purified rDen 3 EDIII protein following subsequent purification step as affinity chromatography will purify all conformers of rDen 3 EDIII protein produced in *E. coli* through histidine tag. Processing of biomass from pilot scale batch fermentation yielded 35 mg (Table 8.2) of highly pure, refolded rDen 3 EDIII protein per liter culture. Though this yield is reasonable to proceed for production of cGMP grade rDen 3 EDIII protein for use in human dengue vaccine studies, efforts are being made to further improve the final yield.

Table 8.2. Purification of rDen 3 EDIII protein from batch fermentation at 100 liter scale.

Purification steps	rDen 3 EDIII protein (mg/l)	% Purity	% Yield
Refolding and affinity chromatography	201.36	90%	100%
Cation exchange chromatography salt based	65.44	95%	32.5%
Cation exchange chromatography pH based	35.69	98%	17.7%

8.3.3 Characterization of rDen 3 EDIII Protein

Purified rDen 3 EDIII protein produced by batch fermentation at 100 liter scale was characterized for its purity and reactivity using SDS-PAGE and Western blot analysis. SDS-PAGE analysis of rDen 3 EDIII protein showed single band corresponding to ~12 kDa (Figure 8.4a, 8.4b).

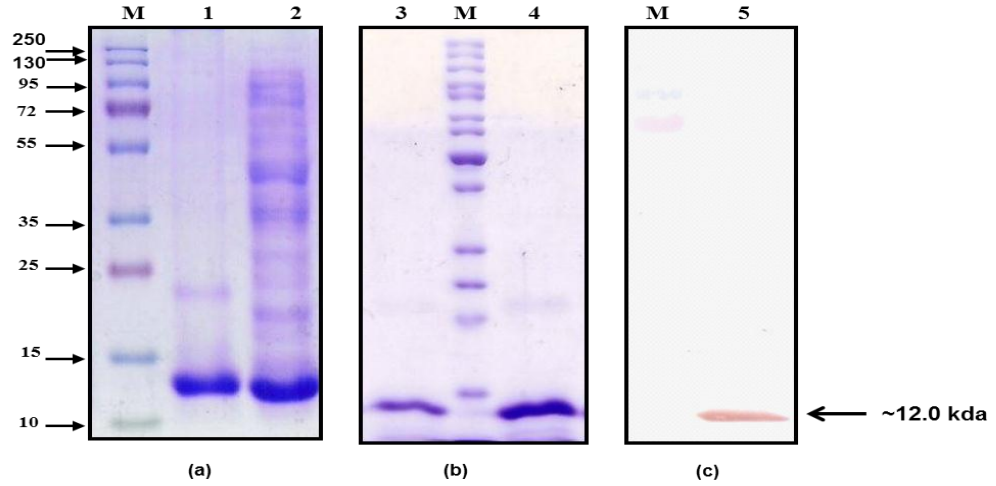

Figure 8.4. (a) Coomassie blue stained SDS-PAGE. The protein band at approximate molecular weight of ~12.0 kDa confirmed the predicted size of the recombinant dengue virus type 3 EDIII protein. Lane M, Molecular Weight Marker (kDa); lane 1, on-column refolded

and IMAC purified rDen 3 EDIII protein; lane 2, Solubilized IBs.(b) Salt and pH based IEX purified rDen 3 EDIII protein. Lane 3, pH based IEX purified protein; lane 4, Salt based IEX purified rDen 3 EDIII protein.(c) Western blot analysis of rDen 3 EDIII protein with polyclonal sera raised against rDen 3 EDIII protein in mice. Lane M, Molecular weight marker; lane 5, Purified rDen 3 EDIII protein with hyper immune sera.

The purified rDen 3 EDIII protein was recognized by anti-dengue antibodies on Western blot showing its reactivity (Figure 8.4c). The characterization of rDen 3 EDIII protein by SDS-PAGE and Western blot confirms that rJEV EDIII protein is highly pure and reactive. It is evident from the results that the rJEV EDIII protein specifically reacted with anti-dengue virus antibody and thus suggests that this protein could also be used for the purpose of diagnosis of dengue virus infection.

8.3.4 Humoral Response in Mice Immunized with rDen 3 EDIII Protein

No dengue vaccine is currently licensed for human use. To investigate whether the produced recombinant dengue protein could induce an antibody response specific for the rDen 3 EDIII protein, a group of mice was immunized with refolded and purified protein formulated with adjuvant. ELISA determined domain III antigen-specific antibody responses after two week of last inoculation. Sera collected from mice were serially diluted and tested for recognition of rDen 3 EDIII by ELISA.

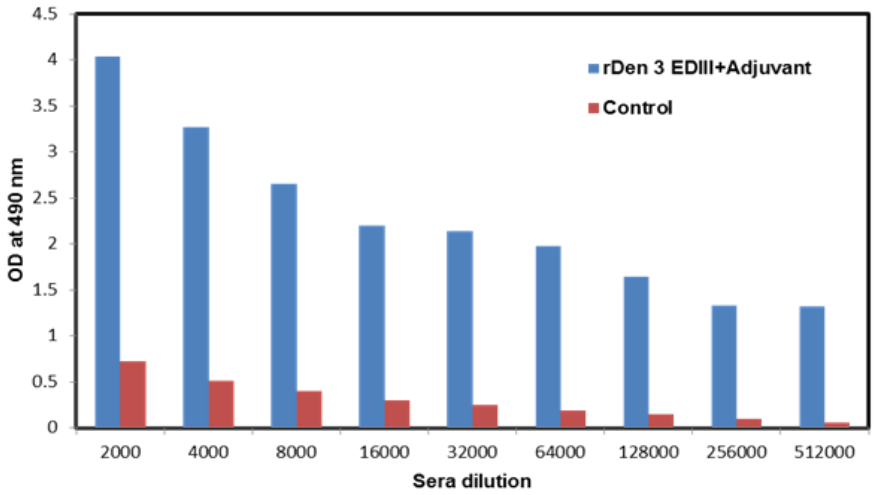

Figure 8.5. Endpoint titers for recognition of rDen 3 EDIII protein. Sera from mice collected at days 56 were tested for recognition of recombinant Den 3 EDIII at various dilutions by ELISA.

The EDIII antigen specific antibody titer was found to be more than 5,12,000 (Figure 8.5). The results showed that rDen 3 EDIII expressed in *E. coli* is able to elicit EDIII-specific antibody responses. Absorbance of healthy mice sera in 1:2000 dilution was 0.840. Figure 8.5 shows absorbance values and the titer of envelope domain III specific protein at 490 nm in immunized mice sera. Formulation of rDen 3 EDIII protein with FCA was highly immunogenic and yielded good ELISA titers. For small molecule or poorly immunogenic, adjuvants can be used for induce an effective immune response or humoral response (Cox and Coulter, 1997). FCA is an important adjuvant for investigators and is regarded scientifically as an effective means of potentiating immune responses in laboratory animals. Our initial evaluations indicated that the rDen 3 EDIII protein in combination with adjuvant elicited humoral immune response in mice.

8.3.5 Plaque Reduction Neutralization Test

The neutralization effect of polyclonal antibodies raised against recombinant Den 3 EDIII protein on the dengue virus type 3 was evaluated by plaque reduction neutralization test in LLC-MK2 cells. Two-fold serum dilutions were tested starting at 1:2 against 100 p.f.u. of dengue virus type 3. A PRNT value of dengue virus type 3 by anti-rDen 3 EDIII antibodies was observed up to a dilution of 1:64 (Figure 8.6). $PRNT_{90}$ titers of the rDen 3 EDIII anti-serum were approximately 1:16 for FCA group sera. High neutralization effect remained up to a dilution of 1:32 (Figure 8.6).

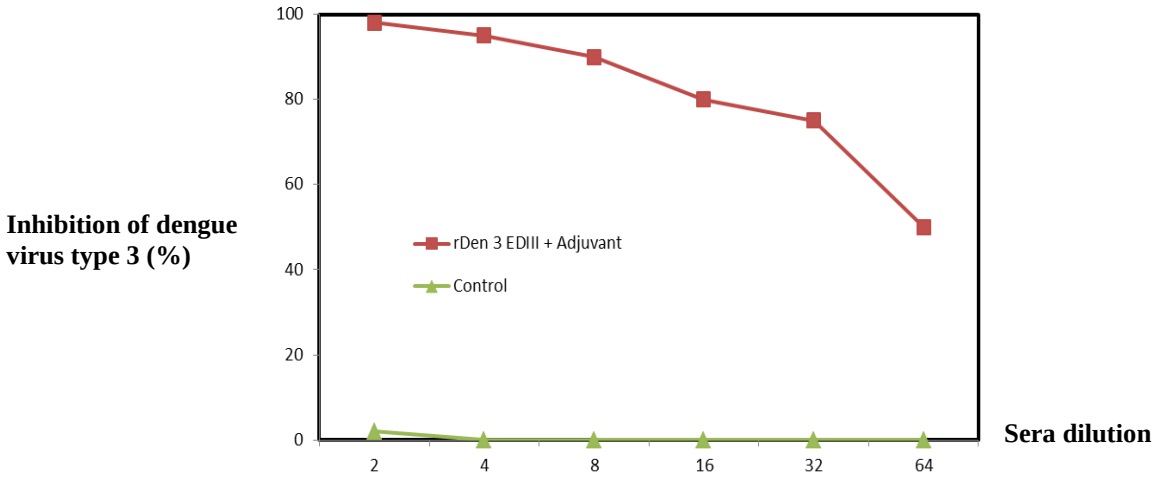

Figure 8.6. Neutralizing ability of sera generated against rDen 3 EDIII protein in mice. High neutralizing antibody titers remained till 1:32 dilution.

The humoral response was characterized by high neutralizing titers by plaque reduction neutralization assay. Neutralizing antibodies can bind to virus and prevent virus from binding to host cell receptors. The polyclonal antibodies raised in BALB/c mice using rDen 3 EDIII protein were found to be neutralizing dengue virus type 3 entry in to LLC-MK2 cells. This implies that the refolding of protein could correctly present the conformational epitopes on the surface of the rDen 3 EDIII protein. The $PRNT_{50}$ titers ≥ 1:10 are considered indicative of protective immunity (Delenda et al., 1994). Our data clearly demonstrate the potential of the rDen 3 EDIII protein to elicit neutralizing, and therefore, presumably protective antibodies against dengue virus type 3.

The results from the present study showed that the purified recombinant dengue virus type 3 EDIII protein could protect host cells from dengue virus type 3 infection. It is likely that the recombinant protein was correctly refolded without loss of its biological function. This result is consistent with the findings of other studies (Zhang et al., 2007) in which dengue virus type 2 envelope domain III protein was expressed in *E. coli* and purified by chromatography. Hence, an ideal subunit vaccine using envelope protein needs to be correctly folded and should be well characterized. This is the first report on the use of a pilot scale produced, refolded and purified as well as fully characterized domain III protein which has been used for the immunomodulatory studies in combination with adjuvant and detailed evaluation of humoral immune response.

In our earlier studies, we described laboratory scale production and affinity chromatography purification under denaturing conditions of rDen 3 EDIII protein for use as diagnostic reagent. The domain III of the envelope protein is particularly an important antigen for vaccine development. Increase in the production to achieve large biomass as well as refolding with further purification to achieve high purity of rDen 3 EDIII protein for application in dengue vaccine studies was necessary. Development of pilot scale fermentation strategy as well as three steps purification with simultaneous refolding as described in this report, made its application more feasible. Further, the rDen 3 EDIII protein was reactive with mice sera in ELISA. These results suggest that the product may be used for development of human dengue vaccine studies.

8.4 CONCLUSIONS

We have developed a 100 liter pilot scale fermentation process for high yield production of recombinant dengue virus type 3 envelope domain III protein from *E. coli*. A simple, three-step purification strategies involving immobilized metal affinity chromatography with simultaneous refolding, salt based cation exchange and pH based cation exchange chromatography, was used to produce rDen 3 EDIII protein that was highly pure, homogeneous and recognized by anti-dengue antibodies. Further, characterization for its immunogenicity and ability to induce neutralizing antibodies established its application more attractive. These findings suggest that rDen 3 EDIII protein in combination with compatible adjuvant is highly immunogenic and can elicit high titer neutralizing antibodies, which proves that refolded and purified rDen 3 EDIII protein can be a potential vaccine candidate. The method described here to produce rDen 3 EDIII protein may also be useful in producing other viral and bacterial proteins in *E. coli* as insoluble form for production at pilot scale.

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CHAPTER 9

PROCESS DEVELOPMENT FOR PRODUCTION OF RECOMBINANT DENGUE VIRUS TYPE 1, 2 AND 4 EDIII PROTEIN AND EVALUATION OF DIAGNOSTIC POTENTIAL OF TETRAVALENT RECOMBINANT DENGUE VIRUS EDIII PROTEIN

ABSTRACT

Dengue hemorrhagic fever and dengue shock syndrome are the severe manifestations of dengue infection. A definitive identification of dengue infection depends on reliable dengue diagnostic tests. There is currently no vaccine to prevent dengue virus infection, which is caused by any one of four serotypes, dengue virus 1, 2, 3 and 4. A dengue vaccine must be tetravalent, because immunity to a single serotype does not offer cross-protection against the other serotypes. To develop diagnostic tests or prophylactic for all dengue virus serotypes, production of EDIII protein of all dengue viruses viz. dengue virus type 1-4 is necessary. In our previous studies we developed high yield scalable process to produce recombinant dengue virus type 3 envelope domain III protein for diagnostic and vaccine studies. In the present study recombinant envelope domain III protein of dengue virus type 1, 2 and 4 was expressed in *E. coli*. Batch and fed-batch fermentation process was developed for dengue virus type 1, 2 and 4 EDIII protein using optimized medium. The proteins were purified by immobilized metal affinity chromatography under denaturing conditions. The physical mixture of envelope domain III proteins of all serotypes was recognized in ELISA with dengue infected human serum samples. These results suggest the EDIII based ELISAs may be useful in epidemiological surveillance and vaccine efficacy trials. This production system may also be suitable for the high yield production of other recombinant proteins expressed in *E. coli*.

9.1 INTRODUCTION

Dengue viruses, of which there are four antigenically distinct serotypes (dengue virus -1, -2, -3 and -4) are mosquito-borne viruses of the *Flaviviridae* family (Lindenbach and Rice, 2003). Dengue virus infection threatens approximately half the global population, and is endemic to over a hundred countries in the tropical and sub-tropical regions of the world. Diagnosis of dengue virus infections, based on clinical presentation, is complicated by its similarity to that of a host of other infectious illnesses including measles, influenza, typhoid, leptospirosis, chikungunya and malaria (Cuzzubbo et al., 2001; AnandaRao et al., 2006). As a result, diagnostic tests are critical in detecting dengue virus infections. For the detection of dengue virus specific antibodies, most of the commercial tests use either a mixture of four inactivated dengue virus particles or four recombinant envelope (ectodomain) proteins

representing four serotypes. Invariably most of these tests fail to differentiate between the antibodies generated against various *flaviviruses* due to the existence of shared antigenic determinants among members of the *Flaviviridae* family such as Japanese encephalitis, tick-borne encephalitis and yellow fever viruses (Batra et al., 2010).

Domain III of envelope protein (EDIII) is considered to be a *flavivirus* species specific antigen and has been used as a diagnostic reagent or for vaccine studies (Simmons et al., 1998; Ludolf et al., 2002; Beasley et al., 2004; Hapugoda et al., 2007; Guzman et al., 2010; Tan and Ng, 2010). To detect antibodies against all four serotypes of dengue virus, it is necessary to use EDIII from four dengue virus serotypes (Simmons et al., 1998). ELISA is a commonly used method for detection of anti dengue IgM and IgG antibodies in dengue infected patient serum samples (AnandaRao et al., 2006; Hapugoda et al.., 2007; Tan and Ng, 2010). *Escherichia coli* is the most commonly used host for heterologous protein production (Sahdev et al., 2007; Huang et al., 2012). Using expression vectors in batch and fed-batch cultivations, recombinant proteins have been successfully expressed in *E. coli*. High-cell-density culture (HCDC) techniques have been developed for use in *E. coli* in order to improve upon the production of recombinant proteins. In HCDC, maximum cell concentrations are most often achieved by using fed-batch processes and various feeding strategies (Shiloach and Fass, 2005). There is currently a need for developing cost-effective, safe and simple diagnostics with sensitivity and specificity as well as suitable vaccine candidate for dengue. In this study, we describe the high yield production and purification of rDen 1, 2 and 4 EDIII protein. Further, we present data demonstrating its utility as a diagnostic reagent by an in-house ELISA in detection of anti-dengue IgM and IgG antibodies in patient serum samples.

9.2 MATERIALS AND METHODS

The materials used for this study are described in chapter 3. The methodologies used for this study are described in materials and methods section (chapter 3).

9.3. RESULTS AND DISCUSSION

9.3.1 Expression of Recombinant Dengue Virus 1, 2 and 4 EDIII Protein

Currently, there is no vaccine to prevent or a drug to treat dengue virus infection. Thus, the availability of reliable diagnostic tools assumes great importance in clinical

management, surveillance and outbreak investigations. In the present study we have produced and purified rDen 1, 2 and 4 EDIII antigens. The major aims of this study were to develop batch as well as fed-batch fermentation process and purification strategies to produce pure protein with high yield. Further evaluation of mixture of EDIII of all serotypes using indirect microwell plate ELISA for detection of both IgG and IgM classes of anti-dengue virus antibodies in human sera was also carried out. The rDen 1, 2 and 4 EDIII gene was previously cloned into pET30a+ and transformed into BLR (DE3) cells. The cells upon induction with IPTG expressed a recombinant protein with a desired molecular weight of ~11.9 kDa rDen 1 EDIII, ~11.7 kDa rDen 2 EDIII and ~11.6 kDa rDen 4 EDIII proteins respectively (Figure 7.1). The appropriate medium for production of these proteins was analysed by using shake flask experiments. For this purpose, LB and modified SB media were tested. The cultures were induced with 1 mM IPTG for 4 h. Modified SB medium resulted more protein yield than LB medium. The final EDIII protein yield at shake flasks using modified SB medium was about 16, 116 and 54 mg per liter of culture for dengue virus type 1, 2 and 4 respectively. However in LB medium, the protein yield was about 11, 60 and 25 mg/l for dengue virus type 1, 2 and 4 respectively.

9.3.2 Production of rDen 1, 2 and 4 EDIII Protein

For *E. coli* or any other cultivation systems, the level of intracellular accumulation of a recombinant protein is dependent on the final cell density. Several recombinant proteins have been successfully produced in recombinant *E. coli* by fed-batch cultivation using various regimes of nutrient feeding resulting in different biomass and production yields. Since the primary goal of fermention research is cost-effective production of recombinant products, it is important to develop a cultivation method that allows the maximization of the yield of the desired product. Although shake-flask production of these recombinant dengue virus envelope domain III proteins was reasonable as mentioned above, the development of a fed-batch process for high yield production of rDen 1, 2 and 4 EDIII protein is required for further studies as a diagnostic reagent or prophylactic purpose. For maximizing the volumetric production, the *E. coli* cells expressing these proteins were grown in a batch and fed-batch cultivation process. Laboratory scale batch fermentation for rDen 2 EDIII protein using modified SB medium, the cultures at DCW 3.72 g/l were induced with 1 mM IPTG and grown for another 4 h. The batch was terminated at a DCW of 7.10 g/l. Further fed-batch

process for rDen 2 EDIII protein production resulted about 21.80 g/l of final DCW at the time of harvest. The real time profile of fed-batch fermentation process for production of rDen 2 EDIII protein is shown in figure 9.1. For rDen 1 EDIII protein production in modified SB medium using batch and fed-batch process the DCW at harvest was about 5 and 16 g/l respectively. In case of rDen 4 EDIII protein using terrific broth medium, the final DCW was 4.56 and 17.34 g/l after batch and fed-batch fermentation respectively.

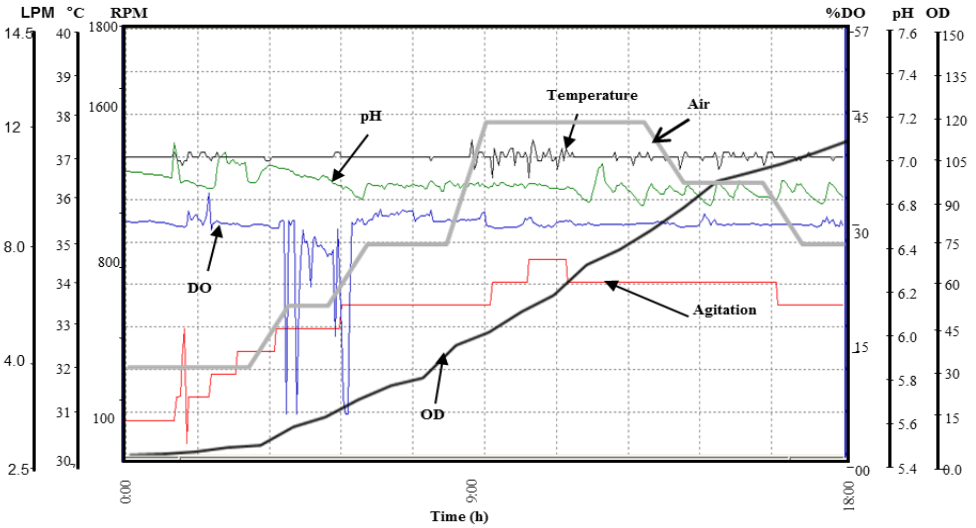

Figure 9.1. Real time profile of fed-batch fermentation process for production of rDen 2 EDIII protein.

Pilot scale fermentation was also carried out to ensure proper scale up from laboratory scale (5.0 liter) to pilot scale (100 liter). Large amount of biomass for purification of rDen 2 EDIII protein used in vaccine studies was necessary. Before going for the pilot scale fermentation, the fermentation conditions and culture medium must be optimized at the small scale using shake flask culture and 5 liter fermentor. Under the optimized conditions, production of rJEV EDIII protein was carried out using 100 liter working volume fermentor with 80 liter modified SB medium. The dry cell weight achieved after batch and fed-batch fermentations for production of these proteins are given in table 9.1. This pilot scale fermentation process for rDen 2 EDIII protein production continued for nine and half hour which comprised of the pre-induction (five and half hour) and post induction (four hour) phase. The DCW reached a final value of 7.04 g/l after pilot scale batch process. The DCW at induction was 3.74 g/l. The successful production of these proteins in fermentor with a high yield was initiated to foster further applications and research with dengue vaccines. The

real time profile of a pilot scale batch fermentation process for rDen 2 EDIII protein production is shown in figure 9.2.

Table 9.1. Production characteristics of *E. coli* expressing rDen 1, 2 and 4 EDIII protein.

Media type	Cultivation mode	Dry cell weight (g/l)	Protein yield (mg/l)
Modified SB	*Batch fermentation[a]	5.16	62
Modified SB	*Fed-batch fermentation[b]	16.30	161
Modified SB	#Batch fermentation[a]	7.10	331
Modified SB	#Batch fermentation[c]	7.04	287
Modified SB	#Fed-batch fermentation[b]	21.80	1040
Terrific Broth	$Batch fermentation[a]	4.56	66
Terrific Broth	#Fed-batch fermentation[b]	17.34	196

[a]At 5.0 liter scale; [b]At 10 liter scale; [c]At 100 liter scale; *for rDen 1 EDIII protein #for rDen 2 EDIII protein; $for rDen 4 EDIII protein.

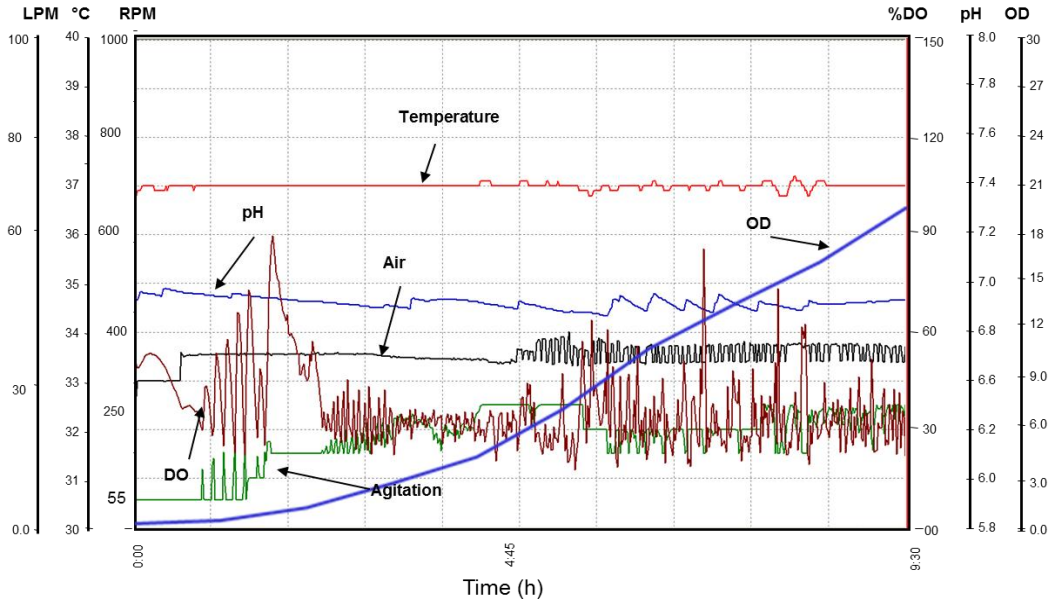

Figure 9.2. Real time profile of pilot scale batch fermentation process for production of rDen 2 EDIII protein.

9.3.3 Purification and Characterization of rDen 1, 2 and 4 EDIII Protein

As the rDen 1, 2 and 4 EDIII protein was insoluble, immobilized metal affinity chromatography was used to purify the protein. After cell disruption and centrifugation,

analysis of the lysate confirmed the presence of these proteins in SDS-PAGE with ~12 kDa protein band (Figure 7.1a). Inclusion bodies were harvested and purified from the induced and lysed cell mass. The inclusion bodies were solubilized in 8M urea containing buffers and purified by affinity chromatography column under denaturing conditions. The real time profile (Time vs OD_{280}) of affinity purification for rDen 2 EDIII protein is shown in figure 9.3. The pilot scale produced biomass was purified using quick scale (70/55) column containing 500 ml Ni-charged streamline chelating AC resin. However lab scale purification were carried out using pre-packed Ni-sepharose columns. The final yield of rDen 1, 2 and 4 EDIII protein after affinity chromatography is given in table 9.1.

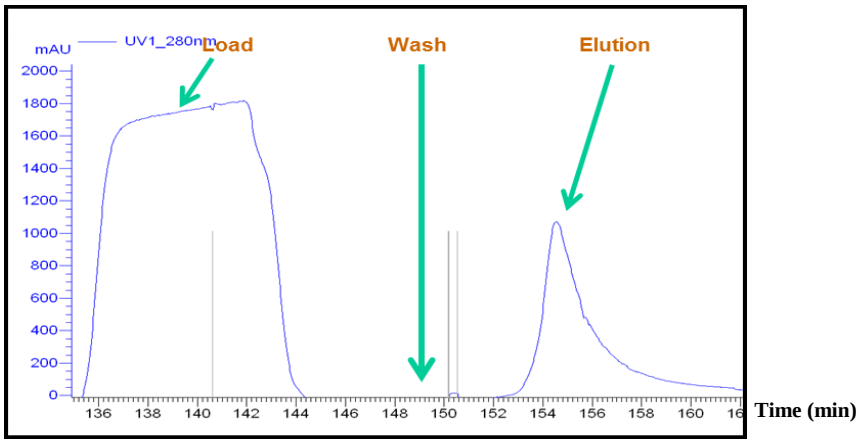

Figure 9.3. Chromatogram showing purification of rDen 2 EDIII protein using IMAC.

The SDS-PAGE analysis of purified rDen 1, 2 and 4 EDIII proteins is shown in figure 9.4. These proteins migrates with molecular weight of ~11.9, ~11.7, ~12.0 and 11.6 kDa on denaturing SDS-PAGE gel for rDen 1, 2, 3 and 4 EDIII protein (Figure 9.4).

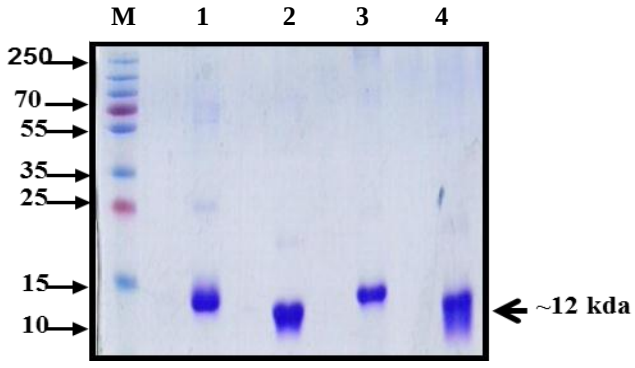

Figure 9.4. SDS-PAGE analysis of affinity purified EDIII proteins. Lane M, MW marker (kDa); lane 1-4, rDen 1, 2,3 and 4 EDIII protein respectively.

The coomassie stained and silver stained gel of pilot scale produced and purified rDen 2 EDIII protein is shown in figure 9.5. From the gel analysis it is evident that purified rDen 2 EDIII protein was highly pure.

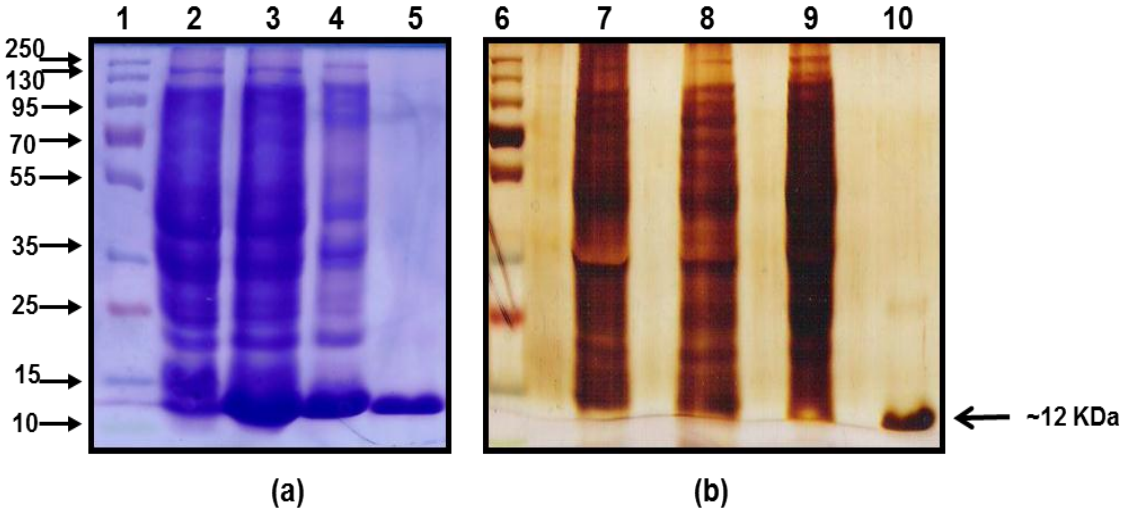

Figure 9.5. (a) Coomassie stained SDS-PAGE gel. (b) Silver stained SDS-PAGE gel. Lane 1 and 6, MW marker (kDa); lane 2 and 7, un-induced lysate; lane 3 and 8, induced lysate; lane 4 and 9, solubilized IBs; lane 5 and 10; AC purified rDen 2 EDIII protein.

9.3.4 Tetravalent Recombinant Dengue Virus 1-4 EDIII Protein as a Diagnostic Reagent

The diagnostic potential of an antigen mixture comprising the EDIIIs of the four dengue virus serotypes for detection of anti-dengue IgM and IgG antibodies was evaluated. For this purpose indirect IgM and IgG microwell plate ELISAs were performed using well characterized 40 human serum samples. All the 40 samples were tested by Pan-Bio IgM and IgG capture ELISA. The OD_{450} values determined by Pan-Bio capture ELISAs and OD_{490} values determined by in-house ELISA for detection of anti dengue IgM and IgG antibodies are given table 9.2 and 9.3. To detect IgM and IgG antibodies for any of the dengue virus serotype 1-4, mixture of EDIII proteins (0.4 µg/ 100 µl/ well) of all serotypes (0.1 µg EDIII protein of each serotype) was used as antigen in in-house microwell plate ELISA to develop diagnostic assay. In IgM ELISA, among 40 samples, 20 samples were positive and 20 samples were negative by in-house ELISA. Out of 40 samples 25 samples were positive and 15 samples were negative by Pan-Bio IgM capture assay. In IgG ELISA, among 40 samples,

20 samples were positive and 20 samples were negative by in-house ELISA. Out of 40 samples 22 samples were positive and 18 samples were negative by Pan-Bio IgG capture assay.

Table 9.2. Range of OD_{450} values in the IgM and IgG capture ELISA for human serum, used to evaluate in-house ELISA.

Capture ELISA OD range[a]	No. of serum samples[b]	No. of serum samples[c]
0.000–0.099	8	6
0.100–0.199	7	12
0.200–0.399	3	2
0.400–0.599	6	5
0.600–0.999	12	8
≥1.00	4	7
Total	**40**	**40**

[a] ELISA OD_{450} values of ≥ 0.20 were considered positive at a serum dilution of 1:100 for IgM antibodies and of 1:1000 for IgG antibodies.

[b] No of serum samples in IgM capture ELISA;

[c] No of serum samples in IgG capture ELISA.

Table 9.3. Range of OD_{490} values in the in-house indirect IgM and IgG microwell plate ELISA for human serum samples.

ELISA OD range[a]	No. of serum samples[b]	No. of serum samples[c]
0.000–0.149	11	8
0.150–0.249	9	12
0.250–0.499	5	3
0.500–0.799	4	8
0.800–0.999	8	3
≥1.00	3	6
Total	**40**	**40**

[a] ELISA OD_{490} values of ≥ 0.25 were considered positive at a serum dilution of 1:100 for IgM antibodies and of 1:1000 for IgG antibodies.

[b] No of serum samples in IgM ELISA; [c] No of serum samples in IgG ELISA.

The data reveal that, both the sensitivity and specificity of a physical mixture of 4 monovalent EDIII antigens in detecting anti-dengue antibodies are comparable to those obtained using Pan-Bio capture ELISA (Table 9.4). Thus tetravalent EDIII protein could be specifically used as a valuable antigen for detection of anti dengue IgM and IgG antibodies in ELISA.

Table 9.4. Comparative evaluation of in-house ELISA with reference toPan-Bio IgM and IgG capture ELISA for detection of anti dengue IgG and IgM antibodies.

Test	n	% Agreement[a]	% Sensitivity[b]	% Specificity[c]
In-house/ IgG capture ELISA	40	95% (38/40)	90% (20/22)	100% (18/18)
In-house/ IgM capture ELISA	40	87% (35/40)	80% (20/25)	100% (15/15)

n, total number of sera tested.

[a] (Number of samples positive by both method + number of samples negative by both methods) /(total number samples) x100.

[b] True positive/(true positive + false negative) x 100.

[c] True negative /(true negative + false positive) x 100.

9.4 DISCUSSION

The immunodominant epitopes in the recombinant dengue virus type 1-4 envelope domain III proteins contribute to a high degree of sensitivity and specificity for diagnosis of dengue infections and to induce neutralizing antibodies. Hence, envelope domain III protein, an independently folding domain that plays an important role in host receptor interaction has become possible diagnostic and vaccine candidate. In the present study, we were able to developed batch and fed-batch process successfully to overexpress envelope domain III protein of all the four serotypes. Further EDIII proteins were purified to homogeneity by affinity chromatography and the purified proteins were successfully tested for the diagnosis of dengue virus infections. Expression of recombinant envelope protein in different expression systems viz. *E. coli*, *Pichia pastoris*, insect cells, transgenic plants was demonstrated recently (Etemad et al., 2008; Block et al., 2010; Tan and Ng., 2010; Chiang et al., 2012). Most of the investigations showed that the yields of recombinant envelope protein have been very low and also difficulties in purification of the envelope protein, which limits its large scale production. Nonetheless, generation of recombinant dengue virus EDIII protein from infected tissue culture insect cells is a laborious and costly,

subjected to batch-to-batch variation making it difficult for routine large-scale production. Production of recombinant EDIII protein in *E. coli* strains is a much cheaper and a simpler procedure (Jaiswal et al., 2004; Zhang et al., 2007; Tripathi et al., 2008; Tan et al., 2010; Tripathi et al., 2011). Several recombinant proteins have been successfully produced in *E. coli* by fed-batch cultivation using various regimes of nutrient feeding resulting in different biomass and production yields (Lee, 1996; Babu et al., 2000; Khalilzadeh et al., 2004; Sahdev et al., 2008; Huang et al., 2012). To enhance the yield, EDIII proteins of dengue virus was produced by batch and fed-batch cultivation processes. The final dry cell weight after fed-batch process for rDen 2 EDIII protein was 21.80 g/l using modified SB medium. The present study demonstrates the feasibility of obtaining pure protein utilizing a single step affinity chromatographic. More than 95% purity of proteins as determined by SDS-PAGE gels has been achieved following a single step purification strategy. The final protein yield of rDen 2 EDIII protein after fed-batch process was 1.04 g/l. The final protein yield of rDen 1 EDIII and rDen 4 EDIII proteins after fed-batch process was 161 and 196 mg/l. The successful production of these EDIII proteins of dengue virus 1, 2, 3 and 4 in fermentor with a high yield was initiated to foster further applications and research with dengue diagnostics and vaccines. The envelope domain III protein, that plays an important role in host receptor interaction has become an attractive candidate, because of the ease of producing small molecules which results in higher yields. In the present study we could successfully developed high yield and scalable production process to produce EDIII proteins of all serotypes of dengue virus.

To test the diagnostic potential of tetravalent EDIII antigen, the ELISA reactivity of a physical mixture of the four EDIII antigens was compared with commercial test using a panel of 40 human sera. A recently reported comparative analysis of several commercial dengue antibody ELISAs has shown that most of these suffer from low specificity (Hunsperger et al., 2009). Therefore, there is a genuine need for a promising test system to detect virus dengue infection. To address the issue of false positives, we included in our study a collection of 20 healthy sera. All these sera were scored as negative samples in both IgM and IgG ELISAs using tetravalent EDIII antigen. The level of observed specificity may stem from the EDIII epitopes predominantly recognizing the dengue virus complex, thereby reducing cross reactivity from antibodies elicited by other non-dengue virus pathogens. In comparison with commercial test used in this study, the in-house ELISA showed 100% specificity, more than 95% accordance and 80% sensitivity. The envelope domain III protein is particularly an

important antigen for vaccine development**,** as it contains multiple serotype specific conformation dependent neutralizing epitopes and host cell receptor recognition site (Guzman et al., 2010). Increase in the production of EDIII proteins for application in diagnostic use as well as for further vaccine studies was necessary. Process development for high yield production as described in this report, made its application more feasible. Further**,** the use of tetravalent EDIII protein as an antigen in in-house ELISA resulted in good agreement with the findings of commercial capture ELISAs. These results show that the product has a promising potential for its use in diagnosis of dengue with comparable sensitivity and specificity.

9.5 CONCLUSIONS

We have developed laboratory scale batch and fed-batch fermentation process for high yield production of recombinant dengue virus type 1, 2 and 4 envelope domain III protein from *E. coli*. Pilot scale fermentation process was also developed to produce rDen 2 EDIII protein. A single step affinity chromatographic purification was used to purify these proteins. Further pilot scale affinity purification was also developed for rDen 2 EDIII protein purification. Our strategy of using a recombinant protein will obviate time-consuming native antigen preparation and the associated biohazard risk. The sensitivity and specificity of tetravalent EDIII protein in detecting antibodies from dengue infected patients render a hope that it may be a good candidate for the development of inexpensive dengue diagnostic tests. The EDIII based ELISAs may be useful in epidemiological surveillance and vaccine efficacy trials. We believe that our approach of producing all four EDIIIs to make a tetravalent recombinant antigen has the potential to lead to the development of an ideal dengue diagnostic test suitable for use, particularly, in the resource-poor regions of dengue endemicity around the globe. This study could also be valuable for the development of tetravalent subunit vaccine for prevention of dengue infections. A similar strategy could lead to the high yield and scalable production of recombinant envelope domain III proteins of other related viruses. Since the expression system uses *E. coli* as the heterologous host, the process can be employed for inexpensive scale-up.

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CHAPTER 10

CONCLUSIONS AND FUTURE ASPECTS

10.1 INTRODUCTION

Recent developments in bioprocess engineering involve large scale production of recombinant proteins. Now a days demand for recombinant proteins has increased because they are being used in therapeutic, prophylactic and diagnostic purposes. The key component of the commercial success of any biopharmaceutical product is the ability to achieve large-scale production. The present challenges for viral diagnostic and vaccine production include the need for scale up, minimization of biohazard risk and production costs. The aim of bioprocess engineering research is the cost effective production of desired recombinant protein by maximizing the productivity. The scale up process of recombinant viral proteins production may be performed by replacing commonly used shake flasks to lab scale and pilot scale fermentations. Recombinant protein purification using minimum possible steps is crucial to meet the required level of purity with low cost. Thus in the present study, lab and pilot scale production, refolding and purification as well as diagnostic and prophylactic potential has been studied using recombinant envelope domain III proteins of JE and dengue viruses. Dengue fever, a mosquito-borne viral disease, has become a major worldwide public health problem, with a dramatic expansion in recent decades. Similarly, Japanese encephalitis (JE) is a major public health problem in Southeast Asia and Western pacific including India. Due to unavailability of effective vaccine or therapeutics, early diagnosis plays a crucial role in patient management. Although early efforts to develop dengue and JE vaccines or diagnostics have focused on conventional approaches, recent approaches focus predominantly on recombinant strategies. The major envelope (E) protein of dengue and JE viruses is considered a lead sub-unit vaccine and diagnostic candidate and virtually all recombinant dengue and JE vaccine approaches are focused on it. Domain III (DIII) of the envelope protein (E) has been identified as the critical region from the perspective of diagnostic and vaccine development. Immunization of animals with EDIII proteins has been shown to elicit neutralizing antibodies to dengue and JE viruses. Studies also showed the diagnostic potential of nonstructural 1 (NS1) protein of dengue and JE viruses. Recent developments and success in recombinant subunit protein vaccine for viral diseases like Hepatitis B, opened new opportunities in dengue and JE vaccine research. Keeping in view of the present scenario of severity and spread of dengue fever and Japanese encephalitis, studies on process development for high yield scalable production and evaluation of new

candidate recombinant dengue and JE antigens for possible diagnostics and vaccine development is the need of the hour. Therefore, in the current research work we intend to produce EDIII protein of dengue and JE viruses at large scale and evaluate the diagnostic potential and immunogenicity.

10.2 SUMMARY AND CONCLUSIONS

In this work, production, purification and characterization of recombinant envelope domain III protein of JE and dengue viruses has been studied. The objective of the present work is to develop high yield and scalable production process for recombinant dengue and Japanese encephalitis envelope domain III proteins in *E.* coli, purification process to achieve high purity and biologically active protein as well as their characterization for use as diagnostic reagent in enzyme linked immunosorbent assay (ELISA) and possible vaccine candidate molecule. Expression of EDIII and NS1 proteins of JE and EDIII proteins of dengue viruses was carried out in recombinant *E. coli.* Development of cost effective and simple culture media as well as appropriate culture conditions is generally favorable for large scale production of recombinant proteins. Optimization of culture media was carried out for enhanced production of EDIII and NS1 protein in *E. coli*. Laboratory scale batch fermentation process in *E. coli* was developed using optimized media and culture conditions. Furthermore, fed-batch fermentation process was also developed in optimized medium. For diagnostic studies, the protein was purified under denaturing conditions using affinity chromatography. The affinity chromatography purified protein was used as an antigen to develop enzyme linked immunosorbent assay (ELISA) to detect antibodies in infected serum samples. In order to take this vaccine candidate for further studies, recombinant EDIII protein was produced employing a pilot scale fermentation process. A three-step purification process comprising of on-column refolding with affinity chromatography, ion-exchange chromatography (IEX) based on salt, and IEX based on pH was developed. Biological function of the refolded and purified EDIII protein was confirmed by their ability to generate EDIII-specific antibodies in mice that could neutralize the virus. These findings suggest that recombinant EDIII protein is highly immunogenic and elicit high-titer neutralizing antibodies. These results establish the application of these proteins to be used for the diagnosis of JE and Dengue virus infection or for further studies in vaccine development.

This process may also be suitable for the high-yield production of other recombinant viral proteins.

Following are the brief summary of the conclusions made in the respective chapters.

10.2.1 *Production of rJEV EDIII protein by batch and fed-batch fermentation as well as evaluation of its diagnostic potential*

The Japanese encephalitis virus envelope domain III gene was cloned into pET30a+ and transformed into BL21 (DE3) cells. The cells upon induction with 1 mM IPTG expressed a recombinant protein with a desired molecular weight of about 13 kDa rJEV EDIII protein. Increase in the production of rJEV EDIII protein for application in diagnostic studies was necessary. In the shake flask culture, the final cell density and cell concentration was found to depend upon the media used. Super broth medium achieved final dry cell weight about 2.55 g/l. However, LB medium produced 1.30 g/l dry cell weight. For maximizing the production, the *E. coli* cells expressing rJEV EDIII protein were grown in a batch and fed-batch cultivation process. Production of the maximum amount of rJEV EDIII protein was attained after 4 h induction with 1 mM IPTG. The final dry cell weight of about 36.45 g/l was attained at ~14 h of cultivation in fed batch mode. In batch cultivation with super broth medium, the dry cell weight at induction and harvest was 2.18 g/l and 4.16 g/l respectively. From SDS-PAGE, the recombinant protein was found associated with the insoluble inclusion body (IBs) fraction. IBs were harvested and purified from the lysed cell mass and solubilized in 8 M urea. The protein was purified by Ni based affinity chromatography under denaturing conditions. The SDS-PAGE gel analysis showed that more than 95% purity has been achieved. The final yield of rJEV EDIII protein was ~910 mg/l, which was increased after fed-batch cultivation to about more than thirty six times in comparison with commonly used shake flask culture with LB medium. This protein was subjected to Western blot assay and revealed that the JEV positive serum sample reacted with rJEV EDIII protein. Evaluation of rJEV EDIII protein by in-house ELISA was carried out to develop detection system for Japanese encephalitis. Comparison of the in-house ELISA using both commercial assays for detection of IgM antibodies to JE revealed comparable sensitivities, specificities and overall agreements. These results show that the product has a promising potential for its use in diagnosis of JE both in laboratory and field condition with comparable sensitivity and specificity. The fed-batch cultivation strategy employed in this study is probably one of the

cost-effective means to enhance cell mass and proteins production. Large quantity of antigenically active recombinant protein, produced by these methods may possibly be used for diagnosis as well as for further studies of immunoprophylaxis in JE virus infection.

10.2.2 Development of a pilot scale production process for rJEV EDIII protein and characterization for its vaccine potential

To conduct vaccine studies with rJEV EDIII antigen, large amount of pure, refolded and biologically active protein was required. Pilot scale fermentations were carried out to produce large amounts of biomass for vaccine studies using modified SB medium. This fermentation process continued for nine and half hours, which comprised of the pre-induction (five and half hour) and post induction (four hour) phase. After fermentation dry cell weight and OD_{600} reached a final value of 6.31 g/l and 19.80 respectively. The DCW at induction was 2.70 g/l. The pilot scale fermentation yielded approximately 2 kg cell paste. Recombinant JEV EDIII protein was refolded by on-column refolding protocol in the presence of glycerol. Three step purification processes comprising of affinity chromatography, ion-exchange chromatography (IEX) based on salt and IEX based on pH was developed. Processing of biomass from pilot scale batch fermentation yielded ~124 mg of highly pure, refolded rJEV EDIII protein from 100 g wet biomass. Pilot scale produced and purified rJEV EDIII protein was characterized for its purity and reactivity using SDS-PAGE and Western blot analysis respectively. SDS-PAGE analysis of refolded and IEX purified rJEV EDIII protein showed greater than 98% purity. The western blot with hyper immune sera raised in mice revealed that the sera reacted with rJEV EDIII protein and confirmed its usefulness. Refolded, purified and characterized rJEV EDIII protein was formulated with FCA adjuvant and used to immunize mice. Sera collected from mice were serially diluted and tested for recognition of rJEV EDIII protein by ELISA. The rJEV EDIII antigen specific antibody titer was found to be more than 2, 56,000. The result shows that rJEV EDIII protein is able to elicit EDIII-specific antibody responses. The neutralization effect of polyclonal antibodies raised against rJEV EDIII protein on the JE virus was evaluated by PRNT in Vero cells. Complete neutralization of JE virus by anti-EDIII antibodies was observed up to a dilution of around 1:16 and high neutralization effect remained up to a dilution of 1:64. These findings suggest that rJEV EDIII protein in combination with compatible adjuvant is highly immunogenic and can elicit high titer

neutralizing antibodies, which proves that pilot scale produced, refolded and purified rJEV EDIII protein can be a potential vaccine candidate.

10.2.3 Production, purification and diagnostic potential of rJEV NS1 protein

The JEV NS1 coding sequence was cloned into pQE30UA and, transformation of the *E. coli* SG13009 strain was carried out. The expressed proteins, following 4 h incubation in the presence of 1 mM IPTG, were monitored by SDS-PAGE. These protein bands with molecular mass of about 44 kDa in the insoluble protein extracts of the recombinant strain corresponded to the predicted mass of JEV NS1 protein. The appropriate medium for rJEV NS1 protein production was analysed by using shake flask experiments. Maximum protein yield was obtained in modified SB medium (2.14 g/l) followed by super broth medium (1.77 g/l). The development of a batch and fed-batch process for high yield production of rJEV NS1 protein is required for further studies as a diagnostic reagent or prophylactic purpose. Batch cultivation with modified SB medium (6.25 g/l) resulted in more DCW in comparison with SB medium (4.30 g/l). For further increasing the production of rJEV NS1 protein per unit volume, fed-batch cultivation using modified SB medium was carried out. The final DCW of about 17.78 g/l was obtained at ~13 h of growth in fed-batch mode. In an effort to obtain target protein in host *E. coli* strain, IBs formation is still considered as a convenient and effective way in recombinant protein production. After cell disruption and centrifugation, IBs were purified. The IBs were solubilized and purified by affinity chromatography under denaturing conditions. From SDS-PAGE profile of protein, more than 90% purity was found to be achieved. Improvement in product yield of more than eight times for fed-batch process as compared to shake flask culture was achieved using modified SB medium. The final rJEV NS1 protein yield after fed-batch cultivation was ~142.16 mg/l. The purified rJEV NS1 protein was tested with mice sera raised against rJEV NS1 protein using Western blot. This revealed that the rJEV NS1 protein could react with anti-JEV NS1 antibody. The usefulness of purified rJEV NS1 protein for the detection of anti-JEV IgM antibodies in human sera and CSF samples was carried out by in-house developed ELISA. The use of this protein as an antigen in ELISA avoids costly and tedious production of viral antigen as well as the inherent biosafety issues. In this study, the high cell density fed-batch cultivation was adopted to increase the protein yield. A simple, one step purification strategy involving metal affinity chromatography was devised to produce rJEV NS1 protein with high purity. The purified

rJEV NS1 protein thus produced has a potential application for detection of anti-JEV IgM antibody. This approach of creating recombinant antigens coupled to over-expression in *E. coli* and simple purification offers a promising alternative option to JE diagnosis with the potential to circumvent the drawbacks of the whole virus antigen based assays.

10.2.4 Production of rDen 3 EDIII protein by batch and fed-batch batch fermentation as well as evaluation of its diagnostic potential

To produce rDen 3 EDIII protein in *E. coli*, EDIII gene was cloned into pET30a+ and transformed into BLR (DE3) cells. The cells upon induction with IPTG expressed a recombinant protein with a desired molecular weight of 12 kDa protein. It was found that 1 mM IPTG and 4 h of post induction time were optimal for the highest expression of this protein. The different culture media resulted in different final OD value and dry cell weight (DCW). Media optimization using batch cultivations resulted in the DCW about 2.10, 2.96 and 4.50 g/l in LB, SOB and SB medium respectively. Further the recombinant cells were cultivated in a fed-batch mode using optimized medium. The DCW at induction and harvest was 15.10 g/l and 22.80 g/l respectively. The final DCW after fed-batch process was found to increase more than seventeen times when compared with that of shake flask culture with LB medium and about nine times more with SB medium. From the localization experiments it was inferred that the recombinant proteins were found associated with the insoluble inclusion body fraction, hence the recombinant proteins were purified under denaturing conditions by Ni-NTA affinity chromatography**.** The batch cultivation yielded 176 mg/l of rDen 3 EDIII protein using optimized medium. The final rDen 3 EDIII protein yield after fed-batch cultivation was 649.24 mg/l. From SDS-PAGE profile of protein, more than 95% purity was found to be achieved. The result of Western blot analysis clearly establishes the use of this protein in the sero-diagnosis of dengue infection. The purified rDen 3 EDIII protein was used for detection of anti-dengue virus antibodies in human sera. A total of 90 serum samples tested for detection of anti-dengue virus IgM and IgG antibodies by both in-house ELISA and Pan-Bio IC test. Agreement, sensitivity and specificity of in-house test with reference to Pan-Bio rapid IC test were 98%, 98% and 100% for detection of anti-dengue virus IgM, and 97%, 100% and 94% for anti-dengue virus IgG antibodies respectively. Large quantity of antigenically active recombinant protein, produced by these methods may possibly be used for diagnosis as well as for further studies of immunoprophylaxis in dengue virus infection.

10.2.5 Development of a pilot scale production process for rDen 3 EDIII protein and characterization for its vaccine potential

Increase in the production to achieve large biomass as well as refolding with further purification to achieve high purity of rDen 3 EDIII protein is required to conduct vaccine studies. Although lab scale production of rDen 3 EDIII protein yielded significant protein levels, the potential requirement for this protein in larger quantity led to its production in pilot scale fermentor. The pilot scale batch fermentation process for production of rDen 3 EDIII protein was carried out using modified SB medium. This fermentation process continued for nine and half hours, comprising the pre-induction (five and half hour) and post-induction (four hour) phase. The DCW and OD_{600} increased continuously and reached a final value of 6.82 g/l and 21.15 respectively. We developed a purification process for this protein to obtain a high quality protein suitable for development of human dengue vaccine studies. The immobilized metal affinity chromatography with simultaneous refolding and cation exchange chromatography based on salt followed by pH was used to obtain highly pure rDen 3 EDIII protein. Processing of biomass from batch fermentation yielded 35 mg of highly pure, refolded rDen 3 EDIII protein per liter culture. To investigate whether the produced recombinant dengue protein could induce an antibody response specific for the rDen 3 EDIII protein, a group of mice were immunized with refolded and purified protein (25 μg protein/dose) formulated with FCA adjuvant. Sera were collected prior to start of immunization and after final immunization from all mice. Mice sera were tested for recognition of rDen 3 EDIII protein by ELISA. Formulations of rDen 3 EDIII protein with FCA were highly immunogenic and yielded about 5,12,000 ELISA titers. The neutralization effects of polyclonal antibodies raised against rDen 3 EDIII proteins on the dengue virus type 3 was evaluated by PRNT in LLC-MK2 cells. Complete neutralization of Dengue virus type 3 by anti-EDIII antibodies was observed up to a dilution of around 1:16 and high neutralization effect remained up to a dilution of 1:32. These results established the potential of pilot scale produced, refolded and purified rDen 3 EDIII protein in dengue vaccine development.

10.2.6 Process development for production of rDen 1, 2 and 4 EDIII protein and evaluation of diagnostic potential of tetravalent rDen EDIII protein.

To develop diagnostic tests or prophylactic for all dengue virus serotypes, production of EDIII protein of all dengue viruses viz. dengue virus type 1-4 is necessary. The rDen 1, 2

and 4 EDIII gene was cloned into pET30a+ and transformed into BLR (DE3) cells. The cells upon induction with IPTG expressed a recombinant protein with a desired molecular weight of ~11.9 kDa rDen 1 EDIII, ~11.7 kDa rDen 2 EDIII and ~11.6 kDa rDen 4 EDIII proteins. From the localization experiments it was inferred that the recombinant proteins were found associated with the insoluble inclusion body fraction, hence the recombinant proteins were purified under denaturing conditions. Recombinant protein production in a laboratory scale is generally performed using complex medium such as LB. However, for producing protein in bioreactor, trials with different media are necessary. High-cell densities were attained with medium enriched in yeast extract and glycerol. For maximizing the volumetric production, the *E. coli* cells expressing EDIII proteins were grown in a fed-batch cultivation process. Shake flask culture, laboratory scale batch and fed-batch fermentation process was developed for dengue virus type 1, 2 and 4 EDIII protein using optimized medium. Further pilot scale batch fermentation was also developed for a dengue virus type 2 domain III protein in optimized medium. After cell disruption, IBs were harvested and purified from the lysed cell mass and solubilized in 8M urea. The proteins were purified by immobilized metal affinity chromatography under denaturing conditions. The SDS-PAGE gel analysis showed that more than 95% purity has been achieved. Reactivity of these proteins was also confirmed using Western blot assay. The final protein yield was 116, 331, 287 and 1040 mg/l after shake flask culture, lab scale and pilot scale batch process and lab scale fed-batch process respectively for rDen 2 EDIII protein. The final protein yield was 161 and 196 mg/l for rDen 1 EDIII and rDen 4 EDIII protein respectively after fed-batch process. To detect any of the dengue virus serotype 1-4, mixture of envelope domain III proteins of all serotypes was used as an antigen in in-house ELISA to develop diagnostic assay. Comparison of the in-house ELISA using this protein with Pan-Bio IgM and IgG capture ELISA for detection of IgM and IgG antibodies to dengue virus revealed 80%, 90% sensitivities and 100%, 100% specificities respectively. The EDIII based ELISAs may be useful in epidemiological surveillance and vaccine efficacy trials.

The conclusions and recommendations of the present study are

1. We have produced and purified biologically functional recombinant envelope domain III protein of the JE and Dengue viruses with high yields. A similar strategy could lead to the production of envelope domain III proteins of other

related viruses. Since the expression system uses *E. coli* as the heterologous host, the process is amenable to inexpensive scale-up. This could be valuable for the development of sub-unit vaccine candidates for prevention of JE and dengue virus infections.

2. The composition of media affected the yield of envelope domain III and NS1 protein production which made its application more feasible. Further batch fermentation process using optimized medium resulted in better yield in comparison to shake flask culture.

3. The fed-batch fermentation strategy employed in this work is probably one of the cost effective means to enhance cell mass and protein production. Taking advantage of high yields obtained by fed batch cultivation, large amount of protein was produced.

4. Pilot scale fermentation process was developed for envelope domain III proteins to produce large amount of biomass for vaccine studies.

5. Laboratory and pilot scale purification process developed to achieve high purity of proteins suitable for diagnostic and vaccine studies. Single step affinity chromatography used for that protein, used as diagnostic antigens and three step purification strategies, for that protein, used for vaccine studies.

6. Further, the use of envelope domain III proteins of JE and Dengue virus and NS1 protein of JE virus as an antigen in microwell plate and dipstick ELISA resulted in excellent agreement with the findings of commercial tests. These results show that the product has a promising potential for its use in diagnosis of JE and dengue both in laboratory and field conditions.

7. For vaccine studies, envelope domain III proteins expressed in *E. coli* were successfully refolded *in vitro* conditions. Characterizations of refolded proteins were carried out to demonstrate the biological activity of the protein.

8. The high neutralizing antibody titers observed in mice immunized with the ED III proteins of JE and dengue virus indicate that the recombinant EDIII protein deserves further study as a potential subunit JE and Dengue vaccine candidate.

9. Envelope domain III proteins formulated with FCA adjuvant was found to be highly immunogenic yielding high titers of neutralizing antibodies.

To summarize, the results of the present study has contributed to the greater understanding of the high yield production of JE and Dengue virus EDIII as well as JEV NS1 protein in *E. coli*. The large scale fermentation and purification strategy may be valuable for developing inexpensive diagnostic test systems and vaccines. Further the ability of envelope domain III protein to induce high titers of neutralizing antibodies with adjuvant pave the way for development of a Japanese encephalitis and dengue vaccine in future. The similar strategy could open up the avenues for development of diagnostic/prophylactic for other related viral disease.

10.3 FUTURE SCOPE OF THE WORK

From the above results it can be concluded that envelope domain III proteins produced at large scale has potential for the use as a diagnostic agent for dengue and Japanese encephalitis. Further studies are required to fully establish the envelope domain III proteins as a JE and dengue vaccine candidate for human use. The followings are the recommendations for the future work.

- Evaluation of recombinant JEV and tetravalent dengue virus 1-4 envelope domain III and JEV NS1 proteins based ELISA with large number of suspected serum/ CSF samples in field conditions.
- Development of rapid immunochromatography (IC) test for JE and dengue using these recombinant proteins.
- Studies on vaccine potential of tetravalent envelope domain III protein of dengue virus serotype 1-4 and JE virus in monkey model.

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